UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA VEGETAL

Recombinant Antimicrobial Polypeptides as Alternative Antibacterial Agents

Ana Isabel Ricacho Gouveia

Mestrado em Biologia Molecular e Genética

Dissertação orientada por:
Prof. Doutor Carlos São-José, Faculdade de Farmácia da Universidade de Lisboa
Profª. Doutora Raquel Sá Leão, Faculdade de Ciências da Universidade de Lisboa

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ABSTRACT

Due to the rapid development of drug resistant bacteria, there has been an effort in the search of new classes of molecules that may substitute or complement conventional antibiotics. Antimicrobial peptides (AMPs) and endolysins have been proposed and explored as two of the most promising alternatives. However, the application of these molecules, *per se*, in a therapeutic context has revealed some limitations that may hinder the antibacterial efficacy of the agents. AMPs are short peptides synthetized by most living organisms as part of antimicrobial defense mechanisms. Their antibacterial mode of action involves perturbation of the bacterial cell envelope, frequently with disruption of the so-called membrane proton-motive force (PMF). On the other hand, endolysins are enzymes encoded by the viruses that infect bacteria, the bacteriophages or phages. These enzymes degrade the peptidoglycan, an essential polymeric component of the bacterial cell wall. The capacity of recombinantly-produced endolysins to degrade the cell wall and cause bacteriolysis is what confers their antibacterial character (enzybiotics). However, it has been shown that, when actively growing in complex media, Gram-positive bacteria may present some tolerance against the lytic action of endolysins. The mechanisms responsible for this tolerance remain unknown, but they seem to depend on an intact PMF. Based on these observations, we have hypothesized that the PMF disturbing character of AMPs could be used to potentiate the bacteriolytic activity of endolysins. To test this idea, selected AMPs were fused to an endolysin targeting *Bacillus subtilis*, a model Gram-positive bacterial species. For one of the AMPs it could be shown that its fusion to the endolysin resulted in a dramatic increase of the bacteriolytic activity under optimal bacterial growth conditions. This effect required though that the fusion proteins acted at the minimum concentration of 1 micromolar. Likewise, the bactericidal activity of one of the fusion proteins showed an increase of 3 orders of magnitude when compared to the unmodified endolysin. Overall, our results point towards the generation of a promising new class of antibacterial agents towards Gram-positive bacteria, named AMPLys, which results from combining the membrane disturbing action of AMPs and the cell wall degrading activity of endolysins in a single agent.

Keywords: Antibiotic Resistance; Antimicrobial Therapy; Endolysin; Antimicrobial Peptide; Enzybiotics.

SUMÁRIO

Devido ao rápido desenvolvimento de bactérias resistentes a múltiplos antibióticos, tem havido um grande esforço no sentido de serem formuladas novas classes de moléculas que constituam uma alternativa viável aos antibióticos convencionais. Os péptidos antimicrobianos (AMPs) e as endolisinas têm sido propostos como sendo duas das alternativas mais promissoras. No entanto, a aplicação destes agentes em contexto terapêutico tem demonstrado algumas limitações que podem prejudicar a sua eficácia antimicrobiana. Os AMPs são pequenos péptidos sintetizados pela grande maioria dos seres vivos, fazendo parte dos mecanismos de defesa antimicrobiana. O seu modo de ação envolve a perturbação do invólucro celular bacteriano, levando frequentemente à dissipação da força proto-motriz (PMF). Por sua vez, as endolisinas são enzimas codificadas por vírus que infetam bactérias, os bacteriófagos ou fagos. São enzimas que degradam o peptidoglicano, um componente polimérico essencial na estrutura da parede celular bacteriana. As enzimas produzidas de modo recombinante conseguem degradar a parede celular, causando bacteriólise, capacidade esta que confere às proteínas o seu caracter antibacteriano (de enzibiótico). Contudo, foi demonstrado que bactérias Gram-positivas em crescimento ativo em meios complexos podem apresentar tolerância à ação bacteriolítica das endolisinas. Os mecanismos responsáveis por esta tolerância ainda não foram elucidados; no entanto, parecem depender de uma PMF intacta. Com base nestas observações, surgiu a ideia de utilizar a ação perturbadora dos AMPs sobre a PMF para potenciar a atividade bacteriolítica das endolisinas. Deste modo, selecionaram-se AMPs para fundir a uma endolisina que tem como alvo *Bacillus subtilis*, uma bactéria modelo Gram-positiva. Para um dos AMPs selecionados, verificou-se que a sua fusão à endolisina resultou num aumento drástico da atividade bacteriolítica em condições ótimas de crescimento bacteriano. Todavia, este efeito só foi observado quando as proteínas atuavam numa concentração mínima de 1 micromolar. Do mesmo modo, a atividade bactericida de uma das fusões apresentou um aumento de cerca de 3 ordens de magnitude quando comparada com a endolisina nativa. Globalmente, os resultados suportam a criação de uma nova classe de agentes antibacterianos contra bactérias Gram-positivas, designada AMPLys, que resulta da fusão de AMPs com ação perturbadora da membrana e de endolisinas que degradam a parede celular bacteriana.

Palavras-chave: Resistência a Antibióticos; Terapia Antimicrobiana; Endolisinas; Péptidos Antimicrobianos; Enzibióticos.

RESUMO

As infeções bacterianas são, geralmente, tratadas com recurso a antibióticos. Desde a sua descoberta, têm sido profusamente utilizados, devido a aliarem a elevada eficácia ao baixo custo de produção. Contudo, devido ao uso excessivo e inapropriado dos antibióticos, tem sido verificado um aumento das bactérias resistentes a estes. Esta situação conduz à progressiva diminuição da eficácia dos antibióticos, pelo que a resistência a antibióticos se tornou uma ameaça a nível global e uma das principais causas de morte. Como tal, sem o desenvolvimento de novas terapêuticas antibacterianas, há a probabilidade de infeções comuns voltarem a ser de difícil tratamento e fatais. No sentido de solucionar esta situação, há uma necessidade eminente de novos fármacos eficazes e com novos modos de ação para o tratamento de infeções bacterianas.

Efetivamente, têm sido realizados esforços de modo a encontrar alternativas aos antibióticos, com baixa probabilidade de desenvolvimento de resistências, novos mecanismos de ação e maior especificidade de ação, anulando deste modo possíveis efeitos secundários sobre as bactérias comensais. Assim sendo, as endolisinas e os péptidos antimicrobianos (AMPs) têm sido propostos como alternativas viáveis e promissoras.

As endolisinas são enzimas produzidas por vírus que infetam bactérias, mais conhecidos por bacteriófagos ou fagos. Estas enzimas são sintetizadas no citoplasma das bactérias hospedeiras durante o ciclo replicativo, atuando na última etapa de infeção. São enzimas responsáveis pela hidrólise do peptidoglicano – o principal constituinte da parede celular bacteriana – promovendo assim a lise da célula hospedeira e a disseminação das partículas virais recém-formadas. Os fagos de cadeia dupla de DNA realizam a lise celular através da ação concertada de pelo menos duas proteínas fágicas, a endolisina e a holina. A holina é uma proteína que oligomeriza na membrana citoplasmática do hospedeiro durante a infeção fágica e, num tempo geneticamente definido, induz a formação de poros. Estes levam à despolarização da membrana, com consequente morte celular. Para a maioria dos fagos estudados, são os poros formados pela holina que permitem à endolisina aceder à parede celular, a qual vai hidrolisar pelo menos uma das principais ligações do peptidoglicano, com consequente lise osmótica das bactérias. O uso das endolisinas num contexto terapêutico foi promovido devido à capacidade destas em induzirem a lise osmótica de suspensões bacterianas. Contudo, verifica-se que quando em condições que promovem o crescimento bacteriano, como as que são encontradas em cenários reais de infeção, as endolisinas podem apresentar atividade lítica reduzida ou mesmo nula.

Os AMPs constituem uma classe de moléculas produzidas por, virtualmente, todos os organismos vivos. São caracterizados, de modo geral, por sequências aminoacídidas curtas (12 a 100 aminoácidos), carga global positiva (apesar de estarem também descritos AMPs com carga global negativa e neutra) e por exibirem carácter anfipático ou hidrofóbico que permitem a sua interação com membranas. Devido a interagirem com as membranas citoplasmáticas, os AMPs levam, muitas vezes, a uma perturbação da membrana e dissipação do potencial energético desta – a chamada força proto-motriz (PMF). Estas moléculas têm sido propostas como uma alternativa promissora relativamente aos antibióticos convencionais, uma vez que demonstram uma ação bactericida rápida, com um largo espetro de ação e apresentam uma baixa incidência de resistência bacteriana. Todavia, há necessidade de melhorar os AMPs para aplicação terapêutica, nomeadamente ao nível da sua estabilidade em condições fisiológicas e relativamente à sua toxicidade (muitos AMPs induzem hemólise nas doses terapêuticas).

Trabalhos desenvolvidos no laboratório de acolhimento têm demonstrado o potencial das endolisinas como enzibióticos contra bactérias Gram-positivas, sobretudo em condições que levam à perda da PMF. Contudo, os mesmos estudos têm mostrado que quando estas bactérias se encontram em condições favoráveis ao seu crescimento e à manutenção da PMF, conseguem oferecer resistência à ação lítica das endolisinas. Na verdade, foi também demonstrado recentemente que a ação de despolarização da membrana mediada pela holina pode ser essencial para potenciar a ação lítica da endolisina. Com base nestas observações, postulou-se que a ação dos AMPs poderia de alguma forma potenciar a ação bacteriolítica das endolisinas, através da fusão de ambas as moléculas. Estes novos agentes que combinam a ação antibacteriana de AMPs e de endolisinas numa mesma molécula foram denominados de AMPLys. Como tal, o principal objetivo deste projeto foi testar o efeito da fusão de dois AMPs selecionados – Salusin-β e Smap – na ação antibacteriana da endolisina LysSPP1 do fago SPP1 de *Bacillus subtilis*.

O trabalho iniciou-se com a fusão genética de cada um dos AMPs selecionados ao N- ou C-terminal da endolisina LysSPP1, recorrendo a técnicas *standard* de DNA recombinante. Este processo permitiu-nos obter e clonar num vetor de expressão as quatro sequências codificantes para as proteínas de fusão: SalβLysSPP1, LysSPP1Salβ, SmapLysSPP1 e LysSPP1Smap.

Apesar de diversas abordagens terem sido tentadas para a otimização da produção das fusões SalβLysSPP1 e LysSPP1Salβ, englobando variações das condições de indução da expressão proteica, variações das estirpes de expressão, ou combinações destas, não foi possível obter estas proteínas na forma solúvel em quantidade suficiente para proceder à sua purificação, condição necessária para desenvolver estudos de atividade antimicrobiana. Felizmente, a produção das fusões SmapLysSPP1 e LysSPP1Smap em condições *standard* permitiu obter quantidades apreciáveis destas proteínas na sua forma solúvel. Deste modo, prosseguiu-se com a sua purificação através de uma cromatografia de afinidade, seguida por uma cromatografia de exclusão molecular. Com este processo foi possível obter as proteínas com elevado grau de pureza. Tendo em conta o objetivo principal deste projeto, o potencial antimicrobiano destes dois AMPLys foi avaliado através da determinação das suas atividades bacteriolítica, bactericida e de inibição do crescimento bacteriano.

A atividade bacteriolítica dos três agentes líticos foi testada em condições subótimas (culturas estáticas) ou ótimas (culturas em agitação orbital) de crescimento das bactérias alvo. Pretendeu-se com estas duas condições obter dois estados energéticos distintos das células, sendo que estas à partida deveriam ser mais suscetíveis à ação dos enzibióticos no primeiro caso. Em condições estáticas a lise promovida pelos AMPLys e por LysSPP1 foi bastante semelhante, apesar de se ter verificado uma tendência de maior suscetibilidade a LysSPP1 quando os agentes foram adicionados em concentrações $\leq$0.5 µM e uma lise mais acentuada promovida pelos AMPLys a concentrações $\geq$1 µM. Quando os mesmos testes foram realizados com culturas em arejamento, o cenário foi bastante semelhante quando os enzibióticos foram testados na concentração de 0.5 µM, apesar de globalmente se registar uma menor lise do que nas condições estáticas, como de resto já era esperado. Contudo, a adição de 1 µM dos agentes líticos LysSPP1Smap e SmapLysSPP1 às culturas em agitação promoveu uma lise celular muito mais rápida e mais extensa, particularmente no caso de LysSPP1Smap, do que aquela promovida pela endolisina nativa. Globalmente, estes resultados sugerem que a eficácia destes AMPLys depende de um limiar de concentração e de um estado energético celular elevado.

Uma vez que a morte celular pode não ser acompanhada de lise das células, foram realizados ensaios que permitiram quantificar a atividade bactericida das proteínas líticas nas condições ótimas de

crescimento. Estes ensaios basearam-se em contagens de unidades formadoras de colónias após um período de incubação com os enzibióticos, permitindo avaliar se existia uma correlação entre a atividade lítica e a atividade bactericida. Verificou-se que os três agentes líticos apresentavam atividades bactericidas semelhantes para concentrações ≤0.5 μM. Para concentrações ≥1 μM, observou-se que a fusão LysSPP1Smap apresentava uma atividade bactericida cerca de 1000 vezes superior a qualquer um dos outros agentes. Concluiu-se assim que, em termos globais, a ação bactericida dos três enzibióticos refletia a sua capacidade lítica. De notar que a alta letalidade da fusão LysSPP1Smap foi registada a concentrações inferior aos MICs reportados para o péptido Smap. Os resultados sugerem, portanto, que existe um efeito sinergístico entre a endolisina e o péptido, em que concentrações subinibitórias do elemento Smap parecem ser suficientes para potenciar grandemente a ação lítica da endolisina.

Finalmente, a inibição do crescimento bacteriano promovido pela LysSPP1 e seus derivados AMPLys foi avaliada através da realização de um ensaio *spot-on-lawn*. Surpreendentemente, a ação inibitória das fusões AMPLys foi muito inferior à de LysSPP1, o que aparentemente contraria os resultados obtidos nos ensaios de atividade bacteriolítica e bactericida. No entanto, estes resultados podem ser explicados com base nas características intrínsecas dos ensaios. Tendo em conta que no ensaio *spot-on-lawn* o crescimento das bactérias ocorre em condições estáticas (em placa de agar semi-sólido) e que há diluição dos agentes devido à difusão, pode-se especular que, neste contexto, as fusões AMPLys atuaram em condições semelhantes aos ensaios de bacteriólise em condições estáticas.

Em resumo, os resultados obtidos neste trabalho indicam que o potencial antimicrobiano das endolisinas pode ser aumentado através da sua fusão com péptidos que destabilizam o ambiente iónico e/ou energético do invólucro celular bacteriano.

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LIST OF ABBREVIATIONS

[K^{2,7,13}]-SMAP-29(1-17) - Smap – derivative of SMAP-29 with 17 residues, with the amino acid residues in the positions 2, 7 and 13 substituted by lysine.

Aa – amino acid

AF – affinity chromatography

Ala – L-Alanine

AMP – antimicrobial peptide

AMPLys – generic name for fusions between antimicrobial peptides and endolysins

Bp – base-pair

BSA – bovine serum albumin

CD – catalytic domain

CM – cell membrane

CW – cell wall

CWBD – cell wall binding domain

DAP – Diaminopimelic acid

DNA – deoxyribonucleic acid

EDTA – ethylenediaminetetraacetic acid

Glu – Glutamic acid

IgG-POD – Immunoglobulin G-Peroxidase conjugate

IPTG – isopropyl β-D-1-thiogalactopyranoside

L-Arg•HCl – L-Arginine monohydrocloride

LB – LuriaBertani medium

MIC – minimum inhibitory concentration

NAG – N-acetylglucosamine

NAM – N-acetylmuramic acid

OD – optical density

OE-PCR – overlap extension polymerase chain reaction

OM – outer membrane

ON – overnight

PCNP – polycationic nonapeptide

PCR – polymerase chain reaction

PG – peptidoglycan

pI – isoelectric point

PMF – proton-motif force

RNA – ribonucleic acid

Rpm – rotation per minute

Salβ – Salusin-β

SDS-PAGE – sodium dodecyl sulphate polyacrilamide gel electrophoresis

SEC – size exclusion chromatography

SMAP-29 – Sheep myeloid antimicrobial peptide of 29 residues

UNITS

CFU/ml – colony forming unit per millilitre

Hr – hours

kDa – kiloDalton (equivalent to kilogram per mol)

Min – minutes

mm – millimetre

MW – molecular weight

nM – nanomolar

Sec - seconds

ºC – Celsius degrees

µg – microgram

ml –millilitre

µl – microlitre

µM – micromolar

µm – micrometre

INDEX

I. INTRODUCTION

I. 1. Antimicrobial Resistance: Public Health at Risk

It is recognized by the scientific and medical communities that the increasing bacterial resistance to antimicrobial treatments is one of the major threats against human health; with infections caused by these pathogens being one the leading causes of death worldwide (Heron, 2017; Troeger *et al.*, 2017; Ventola, 2015). Early after the discovery of penicillin in 1928, it was recognized by Sir Alexander Fleming himself that bacteria could easily, and rapidly, develop resistance when overexposed to the antibiotic (reviewed in Ventola, 2015). The overuse and misuse of antibiotics either in human or in livestock production has led to the emerging of bacterial resistance (Bax *et al.*, 2000; Ventola, 2015). This has been occurring worldwide, culminating in antibiotics with progressively lower efficacy (Ventola, 2015). The antibiotic resistance crisis has also been potentiated by the lack of investment of the pharmaceutical industry in the development of new drugs. Although in the past decades resistance could be partially controlled by the upgrade and modification of pre-existing antibiotics, nowadays it is necessary to develop drugs with new modes of action (Kmietowicz, 2017; Norrby *et al.*, 2005). The chemical modification of already available antibiotics was just a short-term solution, since bacterial cells rapidly acquire resistance to these compounds (Fernebro, 2011). There is a real risk of the world entering a post-antibiotic era, where common bacterial infections may become untreatable and consequently lethal (Norrby *et al.*, 2005; WHO, 2017).

I. 2. Alternatives to Conventional Antibiotics

Due to the rise of multidrug-resistant bacterial strains, it is urgent to invest in research and development of non-conventional antibiotherapy. There are numerous strategies that have shown potential to substitute or complement the current antibiotic drugs. Some of the most promising envisage the use of antibodies, vaccines, bacteriophages, lysins and antimicrobial peptides (AMPs) (Bragg *et al.*, 2018; Czaplewski *et al.*, 2016; Rios *et al.*, 2016; and references therein). These approaches may lead to the development of new drugs, with new modes of action and typically with a narrower spectra of target bacteria than the available antibiotic therapy (Fernebro, 2011).

These alternatives exhibit some interesting features, such as higher specificity for the target pathogens, avoiding therefore collateral effects on commensal bacteria, and less chances of resistance development (perhaps excepting phage therapy in the latter feature). **Antibodies** bind with high affinity and specificity to pathogens (or their virulence factors), promoting their inactivation and clearance by the immune system (Czaplewski *et al.*, 2016; Rios *et al.*, 2016). The potential of **phage therapy** depends on the use of strictly lytic phages as natural predators of bacteria, with the normal progression of a lytic cycle culminating in cell lysis and release of the viral progeny for subsequent rounds of infection (Rios *et al.*, 2016). As phages infect their hosts with high specificity, there is low probability of affecting the benefic microbiota (Bragg *et al.*, 2018). **Phage endolysins** may constitute an alternative antibiotherapy due to their lytic activity. These bacterial cell wall-degrading enzymes were found to be particularly active and exhibit high therapeutic potential against Gram-positive bacteria, since these lack the outer membrane that normally hinders endolysin access to the cell wall in Gram-negative bacteria and mycobacteria (Fernebro,

2011; Rios *et al.*, 2016). Several studies have shown that emergence of resistance to endolysins occurs at much reduced levels when compared to antibiotics (São-José, 2018, and references therein). Another viable alternative is the utilization of **AMPs**, which are ubiquitous peptides produced by almost all living organisms. Most AMPs exhibit bactericidal action due to their ability to disrupt bacterial membranes (Fernebro, 2011; Rios *et al.*, 2016). They are molecules that display a broad spectrum of action, being frequently active against Gram-positive and Gram-negative bacteria, fungi and even viruses (Rios *et al.*, 2016).

In this work we aimed at providing the proof-of-concept of a new class of antibacterial agents, called AMPLys, which results from combining and maximizing the antibacterial properties of two distinct agents, endolysins and AMPs. The fundamental knowledge and rational inspiring the creation of AMPLys are detailed in the next sections.

I. 3. Phage Endolysins as Antibacterial Agents

Endolysins are phage-derived enzymes that cleave peptidoglycan, the major polymeric substance constituting the bacterial cell wall (Vollmer *et al.*, 2008). The most studied endolysins derive from tailed phages and they are responsible for host cell lysis from within in the last phase of the lytic cycle, being essential for promoting viral progeny escape from infected cells (Fernandes & São-José, 2018). Due to their lytic action, endolysins have been intensively explored as possible alternatives to conventional antibiotics (Fischetti, 2010; Nelson et al., 2012).

I. 3.1. Endolysin action in the phage infection context

After the phage replication cycle, the newly formed virion particles must exit the host bacterial cell and, to do that, they must cross the bacterial cell envelop. This is a multilayered structure, typically constituted by a cytoplasmic membrane (CM) and a cell wall (CW), in case of Gram-positive bacteria, in case of Gram-negative bacteria and mycobacteria there is also an outer membrane (OM) surrounding the CW (Willey,J.M., Sherwood, L.M., Woolverton,C.J., 2008).

Double-stranded DNA phages, which include all tailed phages, accomplish bacterial cell lysis through the concerted action of at least two phage-encoded functions, the endolysin and the holin (Catalão *et al.*, 2013). Holins are hydrophobic proteins that oligomerize in CM during phage replication. At the appropriate time for occurring cell lysis (coinciding with a threshold concentration being attained in the membrane), they are triggered to form holes that permeabilize the CM, leading to membrane depolarization and cell death (Young, 2013). Endolysins may be classified in two categories: canonical endolysins (c-endolysins) or exported endolysins (e-endolysins) (Fernandes & São-José, 2016). This classification is based on the way of endolysin translocation to the CW compartment. C-endolysin passage to the CW is through the holin holes, with the latter function being therefore essential for translocation of the lytic enzyme and for establishing the lysis timing (Wang *et al.*, 2000). E-endolysins are conducted to the CW by host cell export machineries, frequently by the bacterial general secretion pathway (Sec system). To avoid premature lysis during phage development, e-endolysin activity is restrained in the CW compartment by mechanisms that depend on the membrane proton motive force (PMF). Therefore, in

these cases holins still have the key role of defining the lysis timing thanks to their PMF-dissipation action (Fernandes & São-José, 2018). Once activated in the CW compartment, endolysins cleave at least one of the four major bonds of the peptidoglycan (PG) network, resulting in cell bursting (osmotic lysis).

I. 3.2. Activity and domain architecture of endolysins

In terms of its structure, endolysins may simply correspond to the globular domain responsible for PG cleavage. These globular endolysins are mostly exclusive of phages infecting Gram-negative bacteria. Phages of Gram-positive bacteria and mycobacteria typically encode for endolysins with a modular architecture, in which a N-terminal module frequently containing one or two catalytic domains (CD) is connected by a flexible linker to a C-terminal cell wall binding domain (CWBD) (**Figure I.1A**) (Nelson *et al.*, 2012; Payne *et al.*, 2012). The CWBD has high affinity to a particular CW component and is responsible for the tight association of endolysins to their substrate (Loessner *et al.*, 2002). A few phages infecting Gram-negative hosts were shown to produce modular endolysins, but in this case with the CWBD and the CD occupying the N- and the C- terminal regions of the enzymes, respectively (Briers *et al.*, 2015, and references therein).

Endolysins typically cleave one or two of the four major bonds in the PG moiety of the bacterial CW, thus compromising its physical integrity. Endolysin CDs can be classified according to their cleavage specificities as N-acetyl-ß-D-muramidases (lysozymes), N-acetylmuramoyl-L-alanine amidases, N-acetyl-ß-D-glucosaminidases (glucosaminidases), endopeptidases, and lytic transglycosylases (Young *et al.*, 2000). Lysozymes, glucosaminidases and lytic transglycosylases act on the glycan strands, breaking the ß-1,4 glycosidic bonds between the N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) repeating unit. Amidases cleave the amide bond connecting NAM to the first amino acid residue of the peptide stem, typically an L-Ala. Endopeptidases cleave within or between the peptide stems. Most of the reported endolysins are classified as muramidases and amidases (**Figure I.1B**).

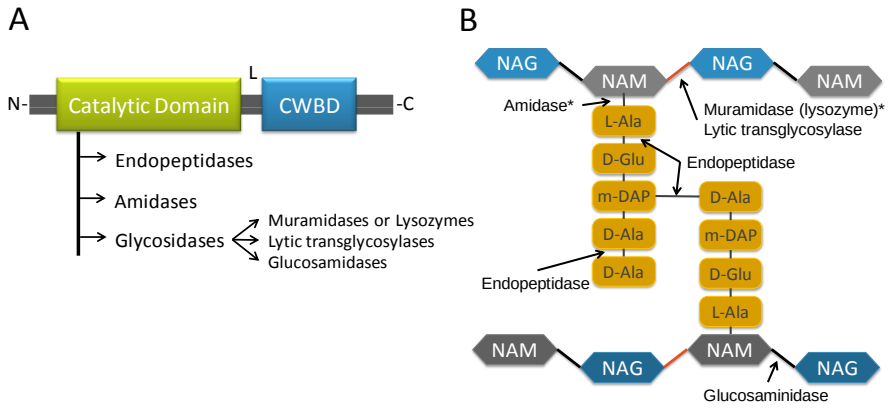

Figure I.1 *Endolysin modular structure and peptidoglycan-degrading activities.*
(A) Generic modular structure found in endolysins encoded by phages infecting Gram-positive bacteria and mycobacteria. The N-terminal catalytic domain(s) (CDs) is(are) attached to a cell wall binding domain (CWBD) by a flexible linker (L). Typically, the N-terminal region harbors one or two distinct CDs displaying one of the five PG cleavage specificities indicated in (B). The C-terminus can carry single or multiple CWBD modules, sometimes multiple copies of the same binding motif. (B) Basic structure of the bacterial cell wall peptidoglycan, with indication of the bonds that are targeted by endolysins. The asterisk identifies the most common enzymatic activities found in endolysins. NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid; Ala, Alanine; Glu, Glutamic Acid; DAP, diaminopimelic acid.

I. 3.3. Exploration of endolysins as enzybiotics

It has been shown that recombinantly-produced endolysins exogenously added to bacteria can lead to rapid osmotic cell lysis. This has been the basis for the exploration of endolysins as antibacterial agents, as part of a broad group of lytic enzymes called "enzybiotics" (Nelson *et al.*, 2001). Several studies published over the past two decades have supported the potential of endolysins as enzybiotics, both *in vitro* and in animal models (Nelson *et al.*, 2012; Pastagia *et al.*, 2013; Roach *et al.*, 2015). However, only a handful of endolysins have made the transition into clinical trials (reviewed in Gerstmans *et al.*, 2018). As referred to above, most frequently the OM of Gram-negative bacteria and mycobacteria blocks the access of the lytic enzymes to the PG layer. Therefore, it is not surprising that the first steps in the development of endolysins as enzybiotics have focused on those targeting Gram-positive bacteria. However, the recent discovery of new enzymes coupled to endolysin engineering strategies has been allowing the application of endolysins to fight Gram-negative bacteria (see below).

The antibacterial potential of endolysins have been proved *in vitro*, for example, against methicillin-resistant and multidrug-resistant *S. aureus* (Fernandes *et al.*, 2012; O'Flaherty *et al.*, 2005; Rashel *et al.*, 2007) and towards vancomycin-resistant *E. faecalis* and *E. faecium* strains (Proença *et al.*, 2012; Yoong *et al.*, 2004). There are also several reports that demonstrate the synergistic effect between endolysins and conventional antibiotherapy (Wittekind & Schuch, 2016). Endolysin action has been evaluated in animal models of pneumonia, endocarditis and sepsis, with focus on efficacy and host immune response (Entenza, Loeffler, Grandgirard, Fischetti, & Moreillon, 2005; Grandgirard, Loeffler, Fischetti, & Leib, 2008; Loeffler, Djurkovic, & Fischetti, 2003; Loeffler, Nelson, & Fischetti, 2001; Loeffler & Fischetti, 2003; McCullers, Karlström, Iverson, Loeffler, & Fischetti, 2007; Witzenrath et al., 2009). From a therapeutic perspective, endolysins will have to act under conditions that are substantially different from those found in their native context, and which may hinder the performance of the lytic agents (São-José, 2018). The great majority of the *in vitro* studies access the bactericidal potential of the enzybiotics when target cells are in conditions that do not support bacterial growth (cells suspended in buffers). When endolysins are tested in complex environments that promote bacterial growth, they usually show diminished or absence of activity. This means that the antibacterial efficacy demonstrated *in vitro* may not be reproduced *in vivo*. In fact, successful application of endolysins in animal models of infection almost always requires that the lytic agents are administrated to animals soon after being inoculated with the bacterial agents (protective rather than therapeutic effect, Oliveira *et al.*, 2018). Some reports have highlighted the relationship between the bacterial cell energy state and the susceptibility to the exogenous action of endolysins (Fernandes & São-José, 2016; Proença *et al.*, 2015).

Although endolysins present important characteristics as antibacterial agents, they may also have some limitations. In addition to the above-mentioned tendency to act poorly against actively growing bacteria, they may exhibit narrow host range, low solubility during large scale production and reduced activity in *in vivo* experiments. To overcome these and other limitations, protein modification and engineering approaches have been followed to improve the therapeutic potential of endolysins.

I. 3.4. Endolysins engineering for improving their enzybiotics properties

The field of enzybiotics has emerged with the exploration of endolysins in its native form. In the past years however, there has been an effort to improve the features of endolysins as enzybiotics. The enhancement of killing activity against bacteria growing in complex environments, the expansion of the spectrum of activity and the ability to target Gram-negative bacteria, are some of the upgraded features. Engineering strategies have also been employed to improve the stability and solubility of endolysins during recombinant production and purification (Gerstmans *et al.*, 2018; São-José, 2018). There are several strategies that can be followed, but the most commonly used involve the construction of chimeric enzymes (chimeolysins) through domain shuffling, domain deletion, fusion to peptides, lysin truncation, or the combination of few of these (Gerstmans *et al.*, 2018; São-José, 2018).

The chimeolysin technology has its basis on the modular character of endolysin functional domains, where the combination of domains from heterologous origins may yield chimeric enzymes with improved characteristics. In an experiment with two *Listeria monocytogenes* phage endolysins, Ply118 and PlyPSA, the individual CDs and CWBDs were swapped to generate chimeras with improved capacity to label and lyse target bacterial cells (Schmelcher *et al.*, 2011). Following this kind of strategy, the resulting chimeolysins may present an expansion of the lytic spectrum, while maintaining the parental enzymatic activities, as it has been demonstrated by fusing the CD of the endolysin of the streptococcal prophage λSA2 to the CWBD of the staphylococcal lytic enzymes LysK and lysostaphin (Becker *et al.*, 2009a). Low *et al*. (2011) found that endolysins carrying CDs with a positive net charge could dispense the presence of CWBD for lytic activity. Actually, deletion of the binding domain in these enzymes resulted even in expansion of the lytic spectrum. Based on these observations, the less negative CD of the pneumococcal endolysin Pal was combined with the high affinity CWBD of LytA (a pneumococcal autolysin). This fusion resulted in a chimera with increased lytic efficiency and with broader host range (Blázquez *et al.*, 2016).

After chimeolysin engineering, the most frequently used endolysin modification involves domain deletions. It could be expected that any deletion of endolysin functional domains would hinder lytic activity. This was in fact observed for some endolysins targeting *B. anthracis* and *S. aureus* (Becker *et al.*, 2015; Porter *et al.*, 2007). However, in certain cases elimination of CWBD was shown to improve endolysin properties, as seen with the staphylococcal endolysin LysK. A derivative containing only the first N-terminal CD of LysK was reported to have enhanced lytic activity against target cells (Horgan *et al.*, 2009). Other truncations resulting in hyperactivity were reported for the streptococcal endolysin PlyGBS (Cheng & Fischetti, 2007). Using an inverse strategy, the addition of an extra CWBD to the *Listeria* phage endolysin Ply500 increased the affinity to the CW, which had a positive effect on lytic activity (Schmelcher *et al.*, 2011).

Fusion of OM-destabilizing peptides to endolysins has been more recently explored as a mean to allow the lytic agents to overcome the OM of Gram-negative bacteria (Briers *et al.*, 2014a; Briers *et al.*, 2014b). The peptides used in this strategy can be polycationic, hydrophobic or amphipathic, all of them promoting OM crossing of the endolysins (Briers *et al.*, 2015). The so-called Artilysins® technology (Briers *et al.*, 2015) relies on the fusion of these OM-destabilizing peptides to endolysins. One striking example of this approach is Art-175, which resulted from the fusion of a natural AMP, SMAP-29, to endolysin KZ144, which targets *Pseudomonas aeruginosa*. This Artilysin exhibited a dramatic increase in

bactericidal activity when compared to the parental endolysin, killing also a wider spectrum of bacterial isolates (Briers *et al.*, 2014a).

I. 4. AMPs as Antimicrobials: Properties and Major Limitations

Antimicrobial peptides (AMPs) are a class of compounds that have been among the most promising alternatives to antibiotic treatment against multidrug-resistant bacteria (López-Meza *et al*., 2011). They are short peptides, produced by virtually all life forms, from microorganisms to humans and superior plants (Mahlapuu *et al.*, 2016). The great majority of AMPs are bactericidal against a broad range of target bacteria, with some acting also against viruses, fungi and protozoa (Ageitos *et al.*, 2016). They can present immunomodulatory properties as well (Fjell *et al.*, 2012). Although AMPs can be organized into distinct classes, they share common features. These include the small size (12 to 100 amino acids), the charged nature (most commonly they are positively charged, although there are examples of neutral and negative AMPs) and the amphipathic or hydrophobic character for interaction with membranes (Raymond Murray Dawson & Liu, 2008).

Regarding their classification, AMPs may be divided into five subgroups based on their amino acid composition and structure: anionic peptides, linear cationic α-helical peptides, cationic peptides enriched for specific amino acids, peptides forming disulphide bonds and peptide fragments of larger proteins (Brogden, 2005).

The unique characteristics of AMPs allow them to easily attack and insert into bacterial cell membranes, with the majority having the potential to damage such membranes (Zhang & Falla, 2006). Regarding the mechanism that leads to cell death, AMPs must first attach to the membrane, which may occur through electrostatic interactions between the cationic peptide and the negatively charged components of the bacterial membrane (Jenssen *et al.*, 2006). Upon binding to membranes, peptides insert into them, with consequent permeabilization. There are several proposed mechanisms of AMP-promoted membrane disruption, the main ones being the ‘barrel-stave model’, the ‘carpet model’ and the ‘toroidal-pore model’, as illustrated in **Figure I.2**.

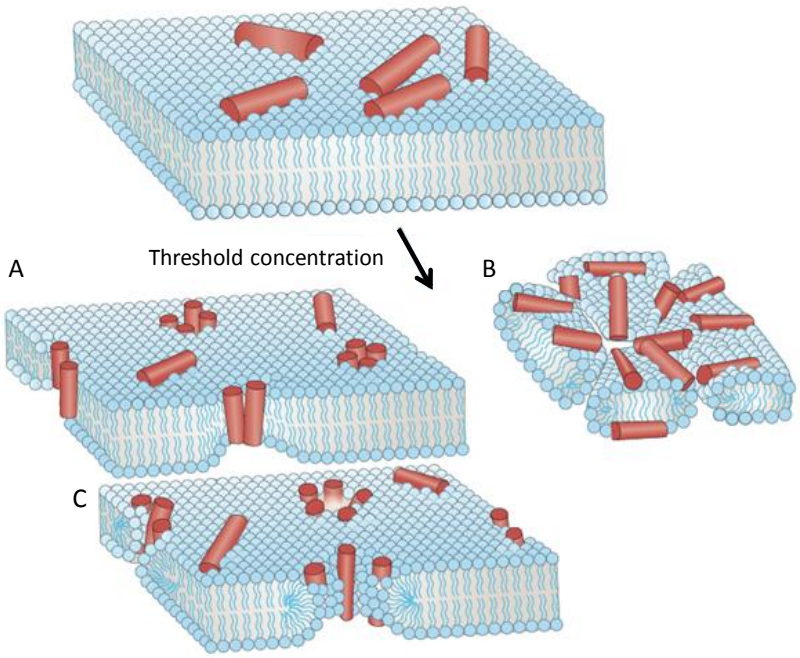

Figure I.2 *Main modes of action of AMPs.*
(A) In the 'barrel-stave model' the peptides insert perpendicularly in the bilayer, associate and form a pore. The hydrophilic region of the peptide faces the pore lumen, while the hydrophobic portion is in contact with the lipid bilayer. (B) In the 'carpet model', the peptides adsorb parallel to the bilayer and produce a detergent-like effect that disintegrates the membrane, leading to the formation of micelles. (C) The 'toroidal pore' mechanism is similar to the 'barrel-stave model', with the peptides also inserting perpendicularly into the bilayer, but instead of forming a pore lined only by the peptides, the pore is lined by both the peptides and by the phospholipid head groups. Adapted from Melo *et al*., 2009.

According to the 'barrel-stave model', a small number of peptides assembles on the cell membrane and then inserts into the lipid bilayer. Further molecules are then recruited and the non-polar domains face the membrane lipids to form a hydrophilic pore that spans the membrane (**Figure I.2A**). In the 'carpet model', the peptides accumulate on the membrane surface, through electrostatic interaction, until a threshold concentration is reached, after which the peptides are oriented parallel to the bilayer and act in a detergent-like manner, culminating in the development of micelles and membrane interruptions (**Figure I.2B**). The 'toroidal pore model' proposes that the AMPs insert perpendicularly to the bilayer, allowing the interaction between the hydrophilic regions of the peptide with the phospholipid head groups of cell membranes. This process induces the curving of the membrane around the peptides, in such a manner that the pore is lined by both the hydrophilic regions of the peptide and the lipid head groups (**Figure I.2C**) (Ageitos *et al.*, 2016; Brogden, 2005; Jenssen *et al.*, 2006; Melo *et al.*, 2009). Despite the different modes of action, they all lead to membrane(s) permeabilization, frequently with dissipation of the cytoplasmic membrane PMF and leakage of cellular contents (Brogden, 2005).

The rapid bactericidal activity of AMPs, their broad range of action and the low incidence of bacterial resistance makes them promising candidates for therapeutic application. Furthermore, the minimal inhibitory and bactericidal concentrations often coincide, which suggests that growth inhibition is generally bactericidal (Zhang *et al.*, 2005). Several AMPs have been reported to inhibit biofilm formation and can effectively kill bacteria in existing biofilms (Batoni *et al*,, 2011; Lashua *et al.*, 2016; Overhage *et*

al., 2008). Despite the existence of numerous AMPs under clinical development for the treatment of various bacterial infections (Mahlapuu *et al.*, 2016), there are several aspects that may affect their therapeutic application.

For AMPs to be used in a clinical approach, they need to be stable under a physiological environment, as they demonstrate susceptibility to proteolytic degradation and loss of activity in the presence of physiological concentration of salts. Additionally, AMPs used in a therapeutic context must not show toxicity to host tissues. Besides these inherent challenges, production costs of the peptides are also a major hurdle to the industrial development and commercialization of AMPs as antibacterial therapeutics (Marr *et al.*, 2006).

There has been an effort to overcome most of the challenges imposed by the use of AMPs as alternative to antibiotic therapy, being the fusion of AMPs with larger proteins one of the most followed strategies. This strategy is reported to enhance protein stability and solubility, and diminishes AMP toxicity (Li, 2011). Another strategy that may be used is the development of AMP synthetic derivatives, as these synthetic molecules can be engineered to avoid cell toxicity and maintain their bactericidal action in the presence of high concentrations of salts (Ageitos *et al.*, 2016).

II. THESIS GOALS

As explained above, there are several obstacles and challenges that may hinder the use of native endolysins and AMPs as antibacterial therapeutics. In this project we have envisaged that by combining the properties of both agents, through protein fusions, we could enhance the killing potency of endolysins under conditions promoting bacterial growth. We hypothesized that the engineered antibacterial agents, the AMPLys fusions, could overcome some limitations of the parental proteins/peptides. As a proof-of-concept, in this work we aimed at the construction and testing of AMPLys fusions designed to target and lyse actively growing *Bacillus subtilis* cells.

III. RESULTS

III. 1. Motivation for AMPLys Construction

Previous studies have highlighted the influence of the energetic state of bacterial cells on their susceptibility to endolysins when these are employed as enzybiotics. Particularly, it was shown that actively growing bacteria in rich media were less susceptible, or even completely refractory, to the lytic action of endolysins added from without. In contrast, under conditions leading to cell energy loss, such as nutrient depletion or presence of PMF-dissipating agents, bacteria were much more prone to lysis by the lytic enzymes (Fernandes & São-José, 2016; Proença *et al.*, 2015). It has been proposed that in the natural context of endolysin action, that is, during phage infection, the PMF-dissipating activity of the holin is responsible for boosting endolysin degrading activity towards the host bacteria cell wall (Fernandes & São-José, 2016).

It has been speculated that the capacity of bacteria to counteract endolysin attack when growing in complex media may be responsible for some limitations observed during *in vivo* application of endolysins (Oliveira *et al.*, 2018; Proença *et al.*, 2015). As described in section I.3.4, several modification or engineering approaches have been followed to potentiate the lytic efficiency of endolysins. Following the observations referred to above, we have hypothesized that the lytic activity of endolysins could be potentiated if combined with the cell envelope disturbing activity of AMPs. Thus, it was postulated that fusion of selected AMPs to endolysins could generate a new class of antimicrobial agents with enhanced bacteriolytic activity, which we have generically called AMPLys.

Based on data from the literature and the Antimicrobial Peptide Database (Wang, 2014), a few AMP candidates for fusing to endolysins were selected and ranked according to the highest number possible of the following acid (criteria: (1) small size (up to 20 amino acids) sequence); (2) minimal inhibitory concentration (MIC) towards *B. subtilis* and *S. aureus* (two Gram-positive model bacteria) under 10 μM; (3) active in complex growth media such as LB or Mueller-Hinton Broth; (4) none or low toxicity to human cells (based on hemolytic or cytotoxic effect); (5) no tertiary or quaternary structures (only linear, secondary structure); (6) no post-translational modifications; (7) amenable to recombinant expression in *E. coli* cells. For this work we selected 2 AMPs, salusin-β (Kimura *et al.*, 2014) and [$K^{2,7,13}$]-SMAP-29 (1–17), with the latter corresponding to a low toxicity version of the sheep myeloid antimicrobial peptide SMAP-29, (Shin *et al.*, 2001). For simplicity, the modified SMAP-29 will be designated here as Smap (see below).

As represented in **Figure III.1**, the two selected AMPs were genetically fused to either the N- or the C-terminus of the *B. subtilis* phage SPP1 endolysin LysSPP1. LysSPP1 is a typical modular endolysin of Gram-positive systems, that is, the enzyme's N-terminus carries a CD responsible for PG cleavage (Amidase_3 family) and the C-terminus a module responsible for CW binding (SH3_3 family) (Fernandes & São-José, 2016).

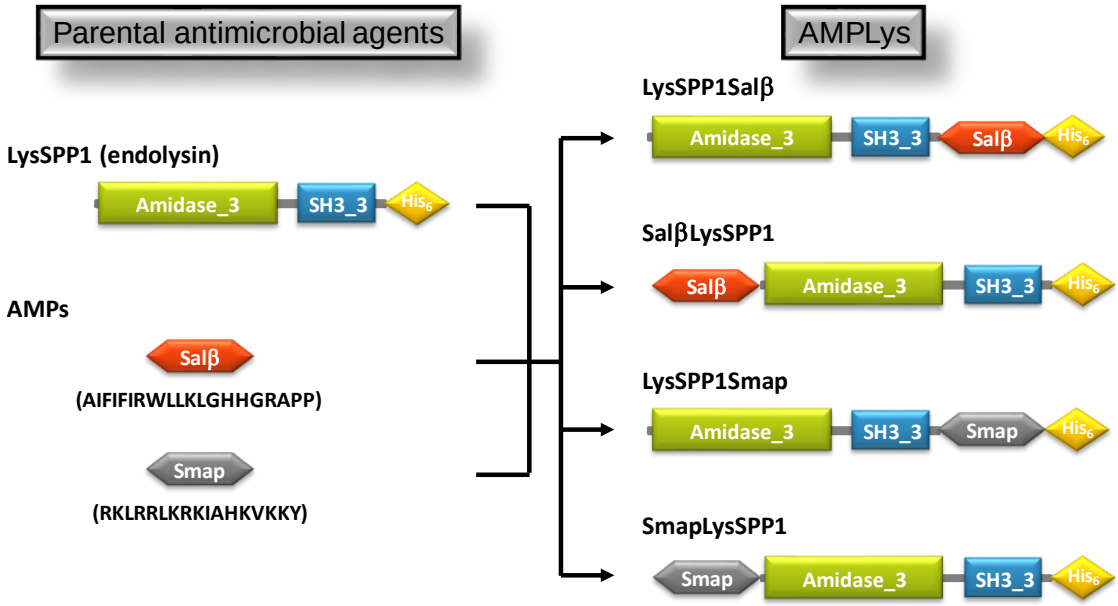

Figure III.1 *Engineering of AMPLys.*

The raw materials for construction of the AMPLys fusions were the endolysin LysSPP1 and the AMPs Salβ and Smap (modified version of the antimicrobial peptide SMAP-29). LysSPP1 is composed of an amidase CD (Amidase_3 family) and a cell wall binding module (SH3_3 family). AMPs aa sequences are indicated in parenthesis. LysSPP1 and AMPLys derivatives carry a C-terminal hexahistidine tag (His_6) for their immunodetection and purification by affinity chromatography (AF).

III. 2. Construction and Production of Salusin-β-Endolysins Fusions

Salusin-β (here sometimes abbreviated to Salβ) is a 20-aa peptide (aa sequence AIFIFIRWLLKLGHHGRAPP) that likely presents a α-helix, monomeric structure. It is detected in several human tissues and fluids and has many biological activities described, namely in the cardiovascular system (Sato *et al.*, 2009; Shichiri *et al.*, 2003; Watanabe *et al.*, 2011). Salusin-β was also reported to have AMP activity against some Gram-positive bacteria by inducing cell membrane depolarization, namely on *B. subtilis* and *S. aureus*, while being much less active against Gram-negative bacteria like *E. coli* (Kimura *et al.*, 2014). The reported salusin-β MICs for *S. aureus*, *B. subtilis* and *E. coli* were 2, 8 and >128 μM, respectively (Kimura *et al.*, 2014).

The AMPLys fusions LysSPP1Salβ and SalβLysSPP1 were obtained by cloning the coding sequence of Salβ and flanking linker sequences (see Supplementary Fig. S1 for aa sequence details) downstream or upstream the LysSPP1 gene carried in the expression vector pIVEX2.3d. The recombinant plasmid pIVEX2.3d::LysSPP1 was available from previous work (Fernandes & São-José, 2016). The expression vector pIVEX2.3d allows *E. coli* production of recombinant proteins C-terminally tagged with a hexahistidine tail (His_6). This tag allowed protein detection by western blot analysis and protein purification by affinity chromatography (AF).

In the first attempt to produce the AMPLys fusions we noticed some cell lysis of the culture producing SalβLysSPP1 after 3 hr post-induction (**Figure III.2A**). Since it was previously shown that expression of the parental protein LysSPP1 did not cause lysis of the *E. coli* expression host (Fernandes &

São-José, 2016), we have assumed that the lysis phenotype was due to the Salβ component of the fusion. Curiously, cultures producing LysSPP1Salβ did not exhibit such lysis phenotype.

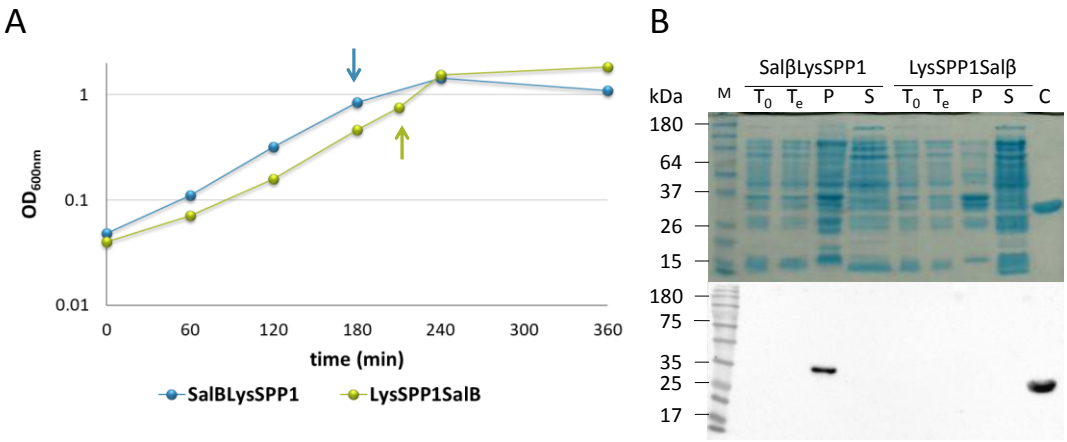

Figure III.2 *SalβLysSPP1 and LysSPP1Salβ production in* E. coli *CG61 strain.*
(A) Growth curves of *E. coli* CG61 derivatives expressing the two AMPLys fusions (the time of protein synthesis induction is indicated by the arrows). (B) SDS-PAGE and western blot analysis of recombinant protein production after 180 min induction. M, protein molecular weight marker; T_0, total protein extract before induction; T_e, total protein extract after induction; P, insoluble fraction of T_e; S, soluble fraction of T_e; C, control for His_6 tag detection. The molecular weight of some bands composing the protein ladders is indicated on the left side.

Analysis of protein production by SDS-PAGE (**Figure III.2B**, top panel) did not allow distinguishing protein bands corresponding to the AMPLys fusions in none of the extracts. The predicted molecular weight of the fusions was 33.99 kDa (SalβLysSPP1) and 34.16 kDa (LysSPP1Salβ). The results pointed therefore for absence or reduced levels of production of the desired proteins. Nevertheless, western blot analysis with an anti-His_6 antibody revealed the presence of a band compatible with SalβLysSPP1 in the insoluble fraction of the protein extracts. LysSPP1Salβ could not be detected in any of the fractions (**Figure III.2B**, bottom panel). The apparent lack of LysSPP1Salβ production could explain the absence of cell lysis of induced cultures when compared to SalβLysSPP1. Resequencing of the LysSPP1Salβ construct revealed a possible nucleotide insertion immediately upstream of the His_6-tag sequence. This frameshift mutation could also explain the lack of signal during immunodetection with the anti-His_6 antibody.

Next, we tried LysSPP1Salβ production using other clones of the construct. In addition, in the following experiments the induction conditions were altered in an attempt to improve protein production: cell cultures were induced at mid exponential growth phase (optical density at 600nm (OD_{600nm}) of about 0.6) and after thermal induction cultures were kept at 37ºC for only half an hour to minimize cell lysis.

SDS-PAGE indicated that two clones expressing SalβLysSPP1 (including the one tested in **Figure III.2**) still produced very low amounts of the AMPLys in these conditions, being detected in total protein extracts only by western blot (**Figure III.3A** and **B**). LysSPP1Salβ production was also not detected in SDS-PAGE analysis of the extracts of two new tested clones. One of the clones however produced a

protein detected by the anti-His_6 antibody, but with an apparent molecular weight smaller than the expected for LysSPP1Salβ (**Figure III.3A** and **B**). In fact, sequence analysis showed that this clone did not carry the expected LysSPP1Salβ sequence. No more studies with the construct LysSPP1Salβ were pursued. Production of the protein extracts in a commercial lysis buffer did not improve the yields of soluble SalβLysSPP1, which continued to be preferentially detected in the insoluble fraction (**Figure III.3C**).

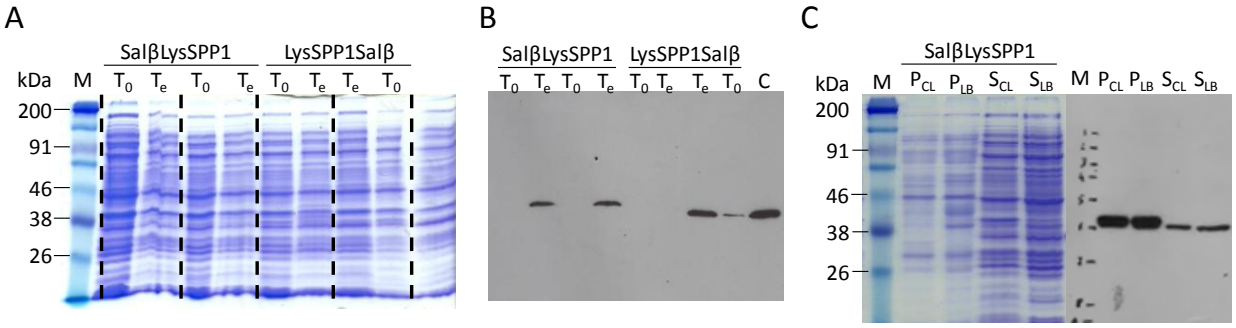

Figure III.3 *SalβLysSPP1 and LysSPP1Salβ production in* E. coli *CG61 strain.*
Two clones of each construct were thermo-induced when cultures reached OD_{600nm} of ~0.6, followed by 30 min at 37ºC. At this point, total protein extracts were produced for SDS-PAGE (A) and western blot analysis (B). In (A) the dashed lines separate the different clones of each AMPLys. M, protein molecular weight marker; T_0, total protein extract before induction; T_e total protein extract after induction C, control for His_6 tag detection. (C) Comparison of SalβLysSPP1 yields in proteins extracts produced in the commercial lysis buffer *Complete lysis-B (2x) EDTA-free* and in the standard, lab prepared lysis buffer (see section VI. 3.1). P_{CL} and S_{CL}, insoluble and soluble protein fractions, respectively, obtained with the commercial lysis buffer; P_{LB} and S_{LB}, insoluble and soluble protein fractions, respectively, obtained using the standard lysis buffer. The molecular weight of some bands composing the protein ladder is indicated on the left side.

The low-level production and insolubility of SalβLysSPP1 could derive from cell toxicity and membrane association caused by the salusin-β moiety. To try improving the production and solubility of SalβLysSPP1, we have expressed the fusion in two mutant strains derived from the widely used *E. coli* BL21(DE3), C41(DE3) and C43(DE3). These strains have proved successful in the heterologous production of membrane and/or toxic proteins (Miroux & Walker, 1996). C43(DE3) is a derivative of C41(DE3) with improved resistance to protein overproduction. We expressed both LysSPP1 and SalβLysSPP1 in these two strains (the native endolysin served as production control). Although no cell lysis was observed with the SalβLysSPP1 construct (as opposed to the results in **Figure III.2A**), there was no improvement in protein production (**Figure III.4**). As expected, LysSPP1 polypeptides could be easily detected both in SDS-PAGE and western blots (**Figure III.4**).

In a final attempt to improve SalβLysSPP1 production and solubility, we expressed the AMPLys fusion in LB medium buffered with sodium phosphate buffer (pH 7.2) and supplemented with either 0.5 M D-sorbitol or 0.2 M L-Arginine monohydrochloride (L-Arg•HCl). These modifications to the LB medium were previously shown to stabilize protein folding and to prevent protein aggregation during protein heterologous production in *E. coli* (Lebendiker & Danieli, 2014; Prasad *et al.*, 2011).

When producing SalβLysSPP1 using buffered LB medium with D-sorbitol we used the expression *E. coli* strains CG61 and C41(DE3) (**Figure III.5A** and **B**). We observed no significant improvement in either production or solubility when compared with previous results. SalβLysSPP1 production in buffered

LB medium supplemented with L-Arg•HCl was only tested with CG61 strain derivatives. In this case we were not able to detect any SalβLysSPP1 both in SDS-PAGE and western blot analysis (data not shown).

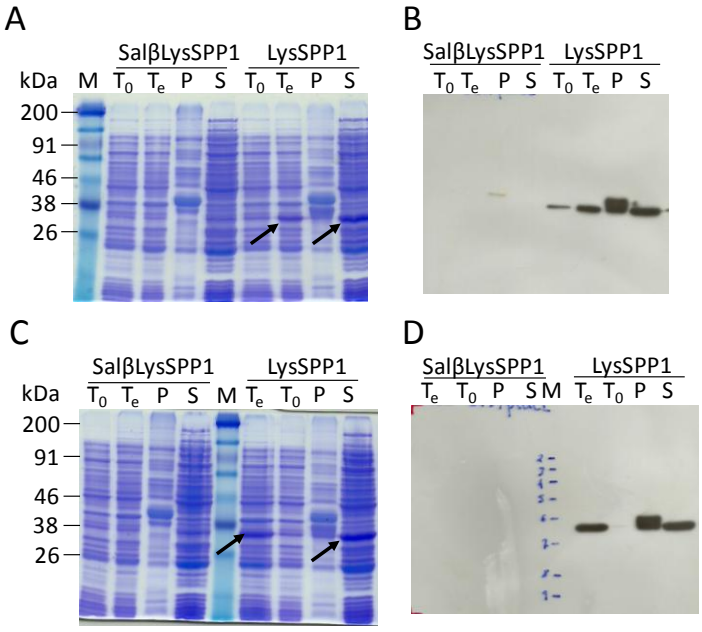

Figure III.4 *SalβLysSPP1 and LysSPP1 production in C41(DE3) (A and B) and C43(DE3) (C and D)* E. coli *strains.* Cultures were IPTG-induced at OD_{600nm} of 0.5-0.6 and protein extracts produced before and 2 hr after induction. These were analyzed by SDS-PAGE (A and C) and western blot (B and D). LysSPP1 polypeptides are indicated by arrows (A and C). M, protein molecular weight marker; T_0, total protein extract before induction; T_e, total protein extract after induction; P, insoluble fraction of T_e; S, soluble fraction of T_e. The molecular weight of some bands composing the protein ladder is indicated on the left side.

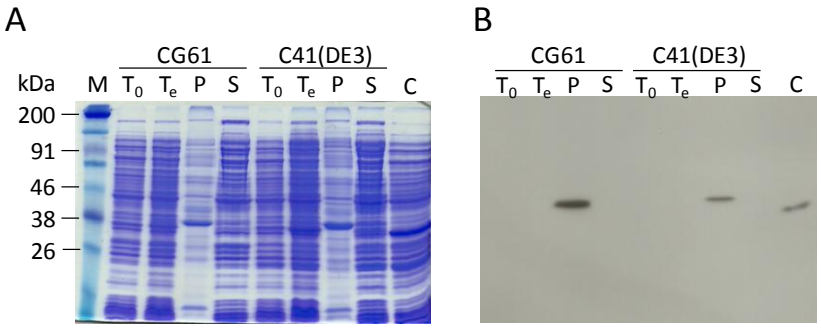

Figure III.5 *SalβLysSPP1 production in modified culture medium.*
Production in *E. coli* strains CG61 and C41(DE3) grown in buffered LB medium supplemented with 0.5 M D-sorbitol. Cells of each strain were grown and induced in the appropriate conditions (see methods) when cultures reached OD_{600nm} of ~0.5. Protein production occurred for 16 hr at 16ºC. Protein extracts were analyzed by SDS-PAGE (A) and western blot (B). M, protein molecular weight marker; T_0, total protein extract before induction; T_e, total protein extract after induction; P, insoluble fraction of T_e; S, soluble fraction of T_e; C, control for His_6 tag detection. The molecular weight of some bands composing the protein ladder is indicated on the left side.

Despite multiple attempts, we could not produce enough soluble amounts of none of the salusin-β-LysSPP1 fusions. Therefore, we next tried to produce the same type of AMPLys fusions using another AMP candidate, in this case Smap.

III. 3. Construction, Production and Purification of Smap-Endolysin Fusions

SMAP-29 (*Sheep Myeloid Antimicrobial Peptide of 29 residues*) is a cathelicidin-related antimicrobial peptide (aa sequence RGLRRLGRKIAHGVKKYGPTVLRIIRIAG). The mature peptide is thought to lack the C-terminal glycine and to be amidated at the C-terminus. This mature form is often referred to as SMAP-28 (Dawson & Liu, 2009). SMAP-29/28 was reported to show broad antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi (Dawson & Liu, 2009; Kalfa *et al.*, 2001; Shin *et al.*, 2001; Tack *et al.*, 2002). SMAP-29 has a disordered configuration in aqueous environments but, in membrane-like environments, at least one segment (residues 8 to 17) seems to adopt a helical structure, whereas the C-terminal half appears to form a hydrophobic, ordered structure (Shin *et al.*, 2001). The antimicrobial activity of SMAP-29 has been attributed to the N-terminal amphipathic α-helix, which inserts in the membrane and causes PMF collapse, whereas the hydrophobic C-terminal region seems to be responsible for its substantial hemolytic/cytotoxic activity (Jacob *et al.*, 2016; Shin *et al.*, 2001).

In a 2001 study, Shin *et al*. evaluated the antimicrobial and hemolytic activity of a variety of SMAP-29 derivatives with altered length and aa composition. One of the derivatives, [$K^{2,7,13}$]-SMAP-29(1-17), corresponded to the first 17 aa of SMAP-29 and carried Gly to Lys substitutions at positions 2, 7 and 13. This version (here referred to as Smap) exhibited strong antimicrobial activity in high salt media, without presenting the hemolytic activity of the parental AMP. The reported MICs for *B. subtilis* and *S. aureus* were 4 and 2 μM, respectively (Shin *et al.*, 2001). Smap was also active against *E. coli* in high salt conditions, although not as efficiently as the parental SMAP-29 (MICs of 6 and 1 μM, respectively) (Shin *et al*., 2001). In theory, this could constitute a limitation for recombinant expression of Smap-endolysin fusions in *E. coli*. However, in one study *E. coli* could be used to express the Artilysin Art-175, which was composed of the native SMAP-29 fused to an endolysin from a *P. aeruginosa* phage (Briers *et al*., 2014a). For these reasons, we choose Smap as an alternative AMP to fuse to LysSPP1.

As done with Salusin-β, the Smap coding sequence with flanking linkers was cloned upstream or downstream the LysSPP1 gene carried in the expression vector pIVEX2.3d, giving rise to fusions SmapLysSPP1 and LysSPP1Smap, respectively (see Supplementary Fig. S1 for aa sequence details). *E. coli* CG61 cells carrying the pIVEX2.3d derivatives were used for production of these new AMPLys (see methods). In contrast to what we had observed with the salusin-β-LysSPP1 fusions, we could maintain *E. coli* growth for 2 hours after induction of protein synthesis without registering significant cell lysis. Production of the new AMPLys fusions was confirmed by SDS-PAGE and western blot analysis, this time with a significant amount of protein detected in the soluble fraction (**Figure III.6**).

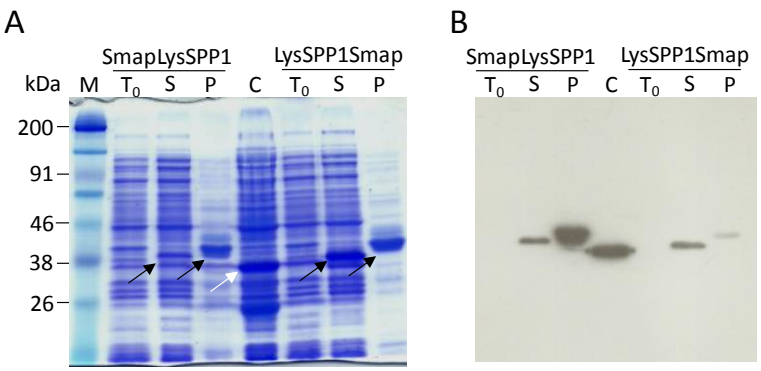

Figure III.6 *SmapLysSPP1 and LysSPP1Smap production in* E. coli *CG61 strain.*
Production of the two AMPLys was analyzed by SDS-PAGE (A) and western blot (B). Cells were thermo-induced when cultures reached OD600nm of ~0.6. Protein production was carried out for 2 hr at 37ºC. M, protein molecular weight marker; T_0, total protein extract before induction; S, soluble fraction of protein extract after induction; P, insoluble fraction of protein extract after induction; C, His_6-tag detection control (LysSPP1 protein). Black and white arrows indicate AMPLys and LysSPP1 polypeptides, respectively. The molecular weight of some bands of the protein ladder is indicated on the left side.

The expected molecular weight of SmapLysSPP1 and LysSPP1Smap was 33.74 kDa and 33.79 kDa, respectively, about 2.7 kDa more than LysSPP1. Curiously, in SDS-PAGE the fusions migrated as polypeptides of ~38 kDa in relation to the protein standard used (BlueStep Protein MWMarkers, Amresco) (**Figure III.6A**). However, the retardation in their electrophoretic mobility relatively to LysSPP1 (31.03 kDa) was compatible with the ~2.7 kDa increment added by the Smap moiety.

The amount of soluble protein of both AMPLys fusions allowed us to proceed to their purification. The His_6-tagged proteins were first captured by affinity chromatography and then further purified by size exclusion chromatography (SEC) (see methods). The SEC column was run with the final, imidazole-free protein buffer. The different steps of the purification process and the level of protein purity obtained in the end were monitored by SDS-PAGE (**Figure III.7**). Although most of the produced protein still sedimented with the insoluble material (compare lanes T_e and S in **Figure III.7A** and **B**), in the end of the affinity step we were able to obtain good quantities of soluble AMPLys. Most of the protein contaminants observed in the affinity peak fractions (lanes "AF Fractions" in **Figure III.7A** and **B**) could be eliminated by the SEC step. Pure fractions were pooled, concentrated and quantified (**Figure III.7C**).

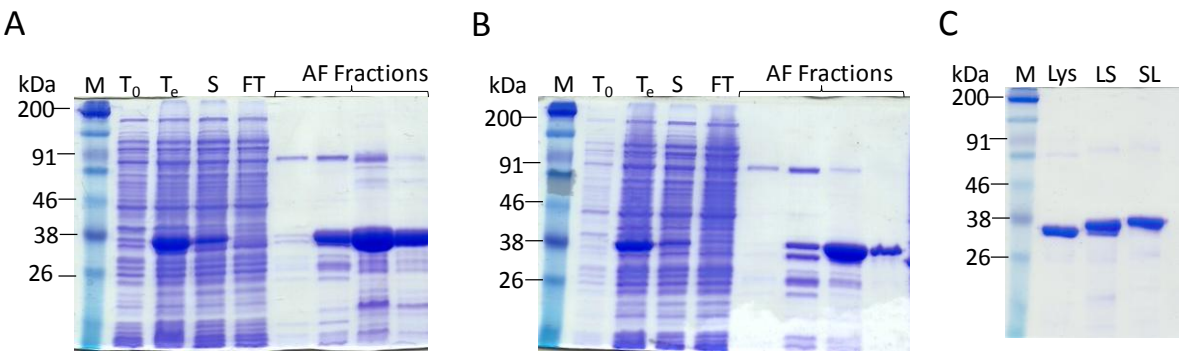

Figure III.7 *LysSPP1Smap and SmapLysSPP1 purification steps.*
SDS-PAGE analysis of the affinity chromatography (AF) purification step of LysSPP1Smap (A) and SmapLysSPP1 (B). The degree of protein purity obtained after the SEC step is shown in C (5 µg protein loaded per lane). M, protein molecular weight marker; T_0, total protein extract before induction; T_e, total protein extract after induction; S, soluble protein extract loaded in the AF column; FT, flowthrough of the AF column; Lys, LysSPP1; LS, LysSPP1Smap; SL, SmapLysSPP1. The molecular weight of some bands composing the protein ladder is indicated on the left side.

III. 4. Bacteriolytic Activity of SmapLysSPP1 and LysSPP1Smap Fusions

As already mentioned, the premise for construction of the AMPLys fusions was that the AMP moiety could potentiate the lytic activity of endolysins when target bacteria are under growth-promoting conditions. Having this in mind, the lytic activity of SmapLysSPP1 and LysSPP1Smap was compared to that of LysSPP1 in different bacterial growth conditions. *B. subtilis* cells were recovered from exponentially growing cultures and then maintained either under static (sub-optimal growth conditions) or aerated conditions (optimal growth conditions) after addition of the lytic agents. In both cases cells were challenged with different concentrations of the proteins (in most assays from 0.25 to 1 µM, roughly from 8.2 to 33 µg/ml) and lysis monitored by taking OD_{600nm} measurements of cell suspensions at regular intervals.

III. 4.1. Bacteriolytic action of AMPLys fusions under static conditions

Under static conditions we did not observe major differences between the lytic activity of the two AMPLys and that of LysSPP1. However, we noticed that up to the concentration of 0.5 µM, the extent of lysis promoted by the native endolysin was consistently slightly higher than that of the AMPLys fusions (only showed for the 0.5 µM concentration, **Figure III.8A**). Interestingly, this trend seemed to change with the next tested concentration (1 µM), since we could observe a slight improvement of the AMPLys lytic activity relatively to LysSPP1 (**Figure III.8B**). Such improvement was also observed for the 2 µM concentration (data not shown).

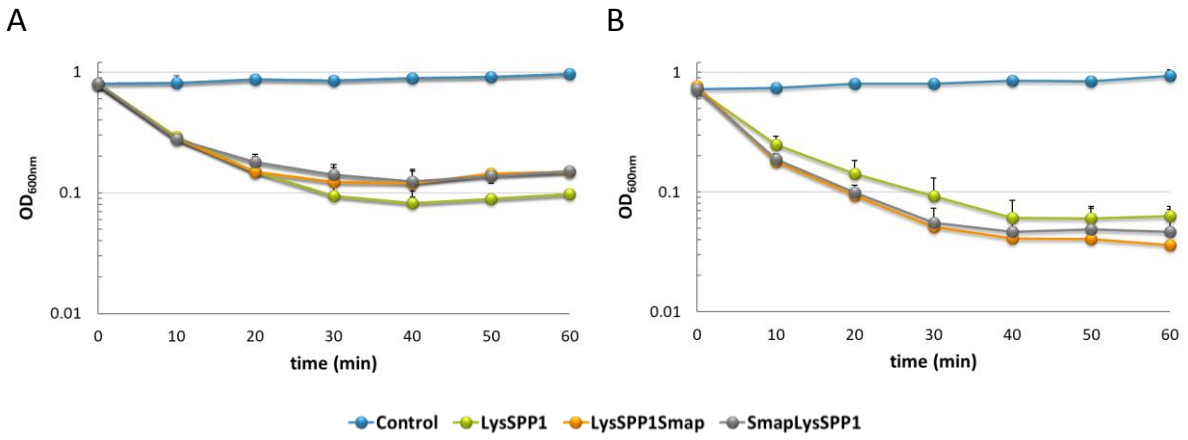

Figure III.8 *B. subtilis lysis under static conditions.*
Cells from exponentially growing cultures were collected and put under static conditions before adding 0.5 µM (A) or 1 µM (B) of LysSPP1 or of AMPLys fusions. Cells lysis was monitored by taking OD_{600nm} measurements at regular intervals. Each curve is the average of at least three independent assays.

We could not see any obvious difference in the lytic activity of both LysSPP1Smap and SmapLysSPP1 at all tested concentrations. As expected, in control assays (maximum equivalent volume of protein buffer added instead of protein) there was no lysis and cells grew very slowly due to the static conditions.

This assay was also carried out with *S. aureus* strain RN4220 and with two *E. coli* strains (TG1 and BL21) under similar conditions. Target cells were harvested from exponentially growing cultures and challenged with 1 µM of each protein. In these assays, none of the three proteins showed the capacity to promote lysis of these cells (data not shown).

III. 4.2. Bacteriolytic action of AMPLys fusions under aerated conditions

We tested next the proteins' lytic activity towards *B. subtilis* cells in conditions favoring cell multiplication, that is, with cells maintained under agitation after challenge with 0.5 µM and 1 µM of each lytic agent. As expected from previous results (Fernandes & São-José, 2016), LysSPP1 lytic activity against aerated cells was decreased when compared to the corresponding assays under static conditions; this was observed for both tested concentrations (**Figure III.9**). When the AMPLys were added at 0.5 µM there was no obvious difference between the lytic capacity of the two fusions. At this concentration, the extent of lysis promoted by LysSPP1 was again slightly higher than that of LysSPP1Smap and SmapLysSPP1 (**Figure III.9A**). Strikingly however, when the AMPLys fusions were tested at 1 µM concentration they exhibited an obvious enhancement of lytic activity compared to the native endolysin. LysSPP1Smap showed faster and more extensive lytic action that SmapLysSPP1, causing a complete clearing of the cultures in about 15 min (**Figure III.9B**).

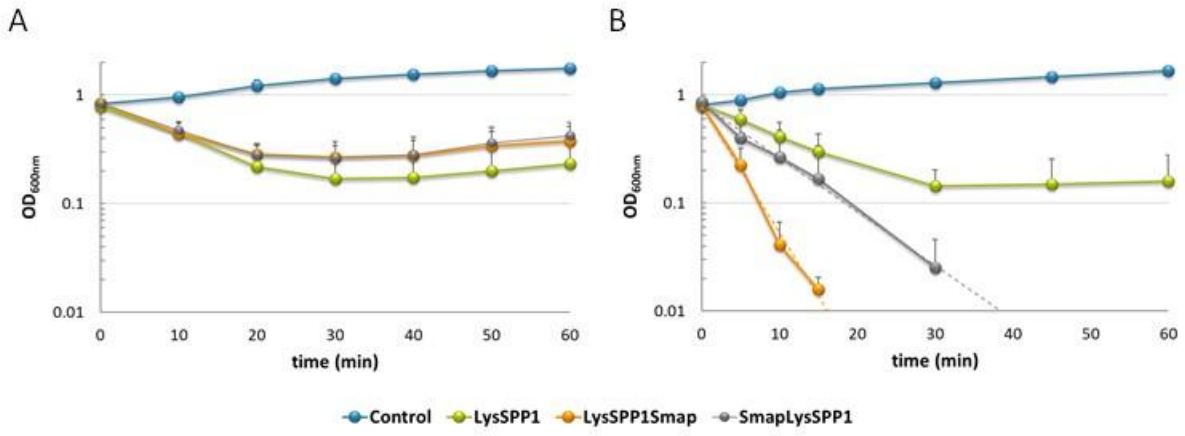

Figure III.9 *B. subtilis lysis under aerated conditions.*
Exponentially growing cultures were challenged with 0.5 µM (A) or 1 µM (B) of native and AMPLys fusions, and lysis monitored at regular intervals. Each curve is the average of at least three independent assays. In panel (B), tendency curves (dashed lines) were added to the LysSPP1Smap and SmapLysSPP1 curves to highlight the rapid and extensive lysis promoted by the AMPLys in these conditions.

In summary, the addition of Smap to LysSPP1 did not seem to improve significantly the lytic activity of the endolysin against cells under sub-optimal growth conditions (lack of aeration). In contrast, under optimal growth (aerated cultures) the AMPLys fusions exhibited a clear enhancement of lytic activity, but this required the agents to act at the minimum concentration of 1 µM. In other words, it seems that the enhanced character of the AMPLys fusions is only observed after they reach a threshold concentration (see Discussion).

III. 5. Bactericidal Activity of SmapLysSPP1 and LysSPP1Smap Fusions

To see if there was a correlation between lytic activity and cell death promoted by the AMPLys fusions under aerated conditions, we determined their impact on cell viability. For that, exponentially-growing *B. subtilis* cells were put in contact with the lytic agents for 30 min and cell viability measured in terms of the log reduction of colony forming units per milliliter (CFU/ml).

When *B. subtilis* cells were challenged with 0.5 µM of each lytic agent, the reduction in CFU/ml was similar in the case of LysSPP1Smap and the native endolysin (about 0.8 log reduction). The SmapLysSPP1 fusion appeared to show a slightly decreased bactericidal effect when compared with the other two agents (only 0.6 log reduction). However, when the enzybiotics were added at the concentration of 1 µM, we observed a drastic increase of about 3 orders of magnitude in LysSPP1Smap bactericidal activity when compared to the native endolysin. On the other hand, SmapLysSPP1 only showed an increase in cell killing of about 0.5 log regarding to the unmodified endolysin (**Figure III.10**). As expected, we have observed an increase of cell counts in the control assays.

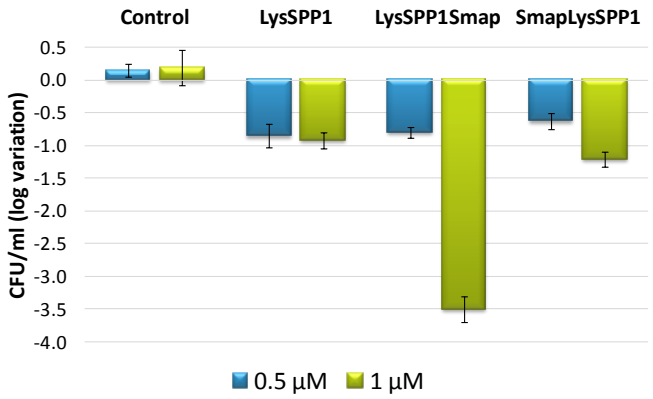

Figure III.10 *Impact of LysSPP1 and its AMPLys derivatives on cell viability under aerated conditions.*
B. subtilis cells (~1 x10^8 CFU/ml) were challenged with 0.5 or 1 µM of the lytic agents for 30 min, after which cell viability was determined in terms of CFU counts. Impact on cell viability is expressed as the logarithmic variation of the CFU/ml values. The results are the average of at least 3 independent assays.

Similar assays were carried out with *S. aureus* cells, but with 2 µM and 4 µM of each lytic agent. There was no measurable bactericidal effect against *S. aureus* even at the highest concentration (data not shown).

Overall, in qualitative terms the bactericidal activity of the three lytic agents correlated well with their bacteriolytic activity under aerated conditions. In the case of LysSPP1, its lytic activity did not differ much when the concentration was altered from 0.5 to 1 µM, and this was reflected on cell viability. For the AMPLys fusions, there was also a good correlation between the results of both assays, although it could be expected a more pronounced increase in SmapLysSPP1 lethality when its concentration changed from 0.5 to 1 µM.

III. 6. Minimum Inhibitory Concentrations of SmapLysSPP1 and LysSPP1Smap in Solid Medium

Finally, we have also compared the ability of the three lytic agents in inhibiting cell growth in soft-agar medium. This effect was evaluated using *B. subtilis* and *S. aureus* cells. Bacterial cells were grown until exponential growth phase and incorporated in LB soft-agar to yield ~10^6 CFU/ml. Serial dilutions of the different agents were spotted on the bacterial lawns to determine the minimum amount producing a clear growth inhibition halo, which provided a rough estimation of the minimum inhibitory concentration (MIC) of each enzybiotic. At least for some lytic enzymes, this spot-on-lawn method has been reported to produce more consistent results than the standard MIC assays in liquid media (Schmelcher *et al.*, 2012).

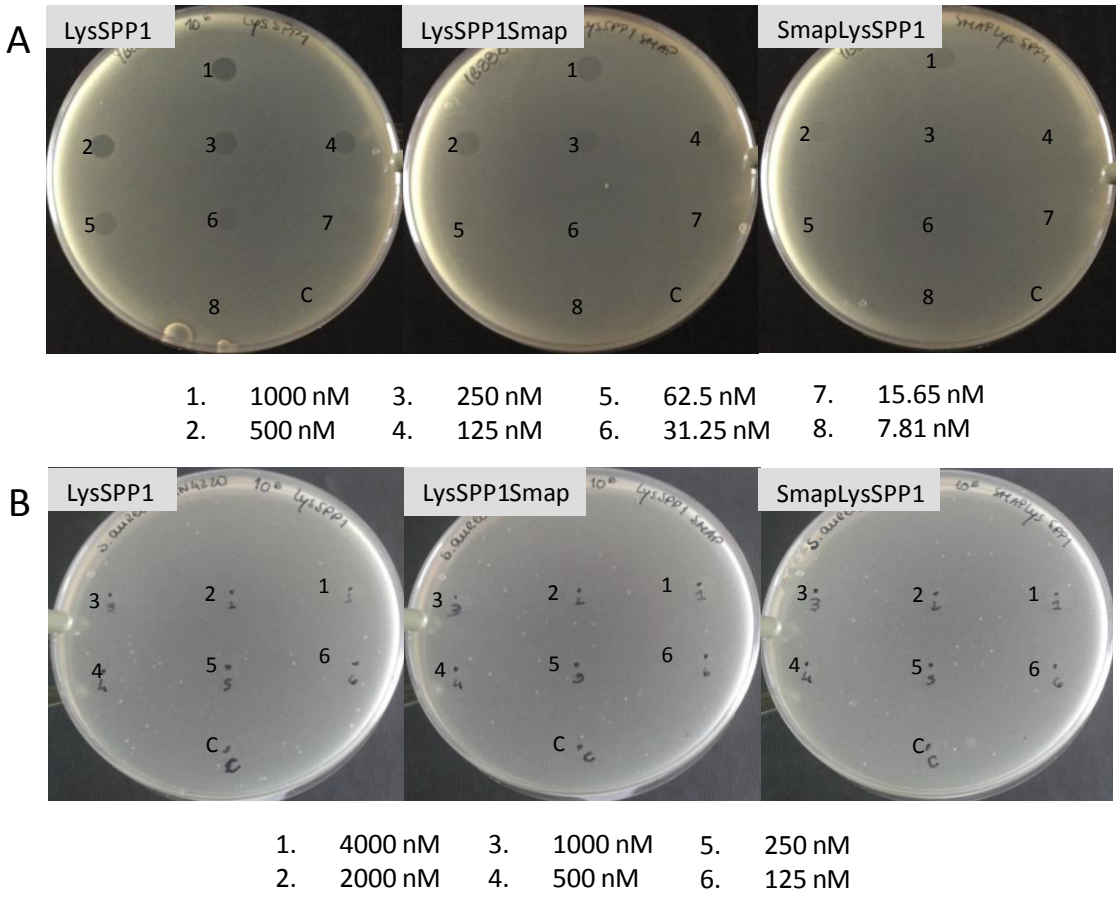

Figure III.11 *MICs of LysSPP1 and its AMPLys derivatives on B. subtilis and S. aureus.*
The three lytic agents were serially diluted to obtain the indicated set of concentrations and then 5 µl samples of each concentration were spotted on a freshly-inoculated lawn of either *B. subtilis* (A) or *S. aureus* (B). Cells were allowed to grow ON to form a dense lawn and growth inhibition evaluated according to the appearance of clear halos. C, agent dilution buffer.

As observed in **Figure III.11A**, the native endolysin showed the highest activity against *B. subtilis*, with the MIC value (first clear halo) established at 125 nM. The MIC values appeared higher for LysSPP1Smap (500 nM) and SmapLysSPP1 (1000 nM). Clearly, these results do not correlate with those obtained in the bacteriolytic and bactericidal assays (see Discussion). When the same assay was carried out with *S. aureus* (**Figure III.11B**), none of the applied lytic agents could inhibit growth, even at the highest concentration (4 µM). It should be noted that in a conventional MIC assay the isolated Smap was reported to have a MIC value of 2 µM against *S. aureus* (Shin *et al.*, 2001), which suggests that the AMP is not able to exert its normal antimicrobial activity when coupled to LysSPP1.

IV. DISCUSSION

The application of endolysins as enzybiotics assumes that the lytic agents should be able to efficiently lyse/kill target bacteria from without, provided that contact to the PG moiety of the CW is allowed. Although there are many reports demonstrating the antibacterial potential of endolysins, the majority of these studies are performed *in vitro* with bacteria washed and resuspended in buffered solutions. These solutions allow cell survival, but not their growth. As highlighted in section I.3.3, the animal studies supporting the efficacy of endolysins are also carried out in particular conditions. Typically, bacteria need to be washed and prepared in physiologic buffers before animal infection and then endolysins must be administered soon after animal inoculation (usually up to 1 hr) for achieving a therapeutic (protective) effect. As argued recently, these assays are a poor approximation of real-life infection scenarios and may not be suitable for proper evaluation of the therapeutic potential of endolysins (Oliveira *et al.*, 2018). Moreover, there is the growing perception that at least Gram-positive bacteria may have PMF-dependent mechanisms to counteract the attack of native endolysins from the exterior (Fernandes & São-José, 2016; Proença *et al*., 2015). As discussed in section I.3.4, the modification of endolysins and the engineering of artificial enzymes are approaches that try to improve the lytic action of these enzybiotics in more complex environments, namely in those supporting active cell proliferation.

As already mentioned, the major goal of this work was to provide the proof-of-concept of a new class of antimicrobial agents, the AMPLys, which are specially designed to amplify the bactericidal effect of phage endolysins against actively growing Gram-positive bacteria. AMPLys result from the coupling of selected AMPs to endolysins, based on the premise that the cell envelope disturbing action of AMPs should potentiate the lytic activity of the lytic agents. In this study we have fused the AMPs **Salusin-β** or **Smap** to either terminus of **LysSPP1**, the endolysin produced by the *B. subtilis* phage.

Despite the multiple conditions tested, we were not able to produce the Salusin-β-endolysin fusions with sufficient yield and solubility. This impossibility to produce the fusions was caused by the specific features of the peptide, since we were able to produce the native protein LysSPP1 in the same conditions. Poor protein production and/or insolubility problems during heterologous expression may result from several mechanisms. Hydrophobic regions may promote protein association with the membrane, with consequent cell toxicity and insolubility. They may also favor protein aggregation, although the latter can also occur as result of the accumulation of high amounts of protein in the cell cytoplasm (Lebendiker & Danieli, 2014). The Salusin-β moiety of the fusions possesses a highly hydrophobic N-terminal region that might have promoted protein aggregation and/or association with the membrane. Modification of the expression hosts and conditions of protein production can be followed to try minimizing protein toxicity and aggregation issues (Leibly *et al*., 2012; Miroux & Walker, 1996; Prasad *et al*., 2011). Unfortunately, our attempts with the *E. coli* expression hosts C41(DE3) and C43(DE3) and with the modified growth media (buffered LB with D-sorbitol or L-Arginine) have proved unsuccessful.

As the several attempts to produce the Salusin-β fusions failed, we changed our focus to a new AMP, Smap, a non-hemolytic derivative of the sheep myeloid peptide SMAP-29 (Shin *et al*., 2001). The native SMAP-29 had been previously fused to an endolysin from a Gram-negative infecting phage to promote translocation of the bacterial outer membrane. The fusion, Artilysin Art-175, could be successfully produced in *E. coli* (Briers *et al*., 2014a). Accordingly, using the Smap derivative, we were able to

produce and purify the AMPLys fusions SmapLysSPP1 and LysSPP1Smap with good yield using standard conditions.

Having our goal in mind, we have evaluated the antimicrobial potential of LysSPP1 and of its AMPLys derivatives against *B. subtilis* under suboptimal or optimal growth conditions, that is, under non-aerated (static) or aerated (orbital shacking) conditions. With static cultures we did not observe substantial differences between the lytic activity of LysSPP1 and AMPLys fusions, apart from a slightly improved lysis of the latter at the higher tested concentrations. Lytic action against cells under aeration conditions was also similar for the three agents when tested at the 0.5 μM concentration, with the lysis extent decreasing compared to static conditions. This agrees with previous results showing that *B. subtilis* cells under robust growth are less susceptible to LysSPP1. Remarkably however, at 1 μM concentration we observed a dramatic increase in lysis promoted by both AMPLys fusions, whereas that of LysSPP1 remained almost unaffected. The lysis increment was particularly striking with the fusion LysSPP1Smap, which completely cleared cultures in less than 15 min. Therefore, the results demonstrate that AMPLys can display much higher lytic activity than parental endolysins when target bacteria are under optimal growth conditions. However, in the particular case of the AMPLys fusions here analyzed, it appears that a clear Smap-mediated enhancement of LysSPP1 activity requires two conditions: the AMPLys concentration must be above a certain threshold (around 1 μM) and *B. subtilis* cells must be under aerated growth.

The existence of a threshold concentration above which AMPLys activity abruptly increases is consistent with the mode of action of AMPs. Typically, to exert their bactericidal effect AMPs must insert and accumulate in the bacterial cell membrane until they reach a critical concentration that leads to membrane permeabilization (Melo *et al*., 2009). A decrease of several orders of magnitude in CFU counts as response to small increments of the concentration of SMAP-29 was previously described (Lin *et al*., 2010). Likewise, the observation that AMPLys lytic activity is much potentiated under aerated conditions may also be related to the nature of AMP interactions with the bacterial cell envelope. Under optimal aerobic growth the *B. subtilis* cytoplasmic membrane is fully energized (Calamita *et al.*, 2001), which might favor the electrostatic interactions that must be established between AMPs and the cell envelope. In addition, the orbital agitation of cultures may help AMPLys distribution and facilitate its interaction with cells.

Given that cell death can exist without occurring cell lysis, we have also evaluated the bactericidal capacity of the AMPLys fusions in terms of CFU/ml reduction, under aerated conditions. The killing effect of the AMPLys fusions and of LysSPP1 was similar (between 0.6 and 0.8 log reduction) when the agents were added to cultures at 0.5 μM concentration. With 1 μM enzybiotic concentration the bactericidal activity of LysSPP1Smap (3.5 log reduction) was much higher than that of the other two agents (up to 1.3 log reduction). Therefore, the bactericidal potency of the three enzybiotics essentially correlated with their lytic activity (see Results). LysSPP1Smap was highly bactericidal at a concentration much lower than the MIC reported for the Smap peptide on *B. subtilis* (4 μM, Shin *et al*., 2001). This suggests that sub-MIC concentrations of Smap are sufficient to potentiate LysSPP1 activity, hinting for a synergistic killing effect between the peptide and endolysin moieties composing the AMPLys agent. Interestingly, none of the AMPLys fusions could cause measurable cell death of *S. aureus*, even when tested at a high concentration (4 μM). The fact that the MIC reported for de isolated Smap against *S. aureus* was 2 μM (Shin *et al*., 2001) seems to indicate that the peptide loses its normal anti-staphylococcal

activity when fused to LysSPP1. However, we should keep in mind that standard MIC assays are carried out under particular conditions and therefore comparisons between different types of experiments should be taken with care. For example, MICs are usually determined with lower bacterial cell inputs (typically 10^5 or 10^6 CFU/ml) when compared to the bacteriolysis and bactericidal assays described in this work (initial cell input of ~10^8 CFU/ml). In addition, the MIC values of the isolated Smap were determined in static conditions (Shin *et al*., 2001), as normally done in standard MIC assays.

We have also evaluated the capacity of LysSPP1 and AMPLys fusions to inhibit bacterial growth by spotting serial dilutions of each protein, starting from 1 µM concentration, on soft agar plates with incorporated *B. subtilis* cells. Surprisingly, and in apparent contradiction to the bacteriolytic and bactericidal assays, the growth inhibition capacity of the fusion proteins was inferior to that of the native endolysin, which showed the lowest "MIC" value. Such discrepancy between different assays used to directly or indirectly evaluate enzybiotics lytic activity has been previously reported (Becker *et al*., 2009b; 2015; Kusuma & Kokai-Kun, 2005). In fact, the bacteriolytic/bactericidal assay conditions that revealed the superior activity of AMPLys (particularly of LysSPP1Smap) are very different from those of the spot-on-lawn experiments. In the first case, the enzybiotics at 1 µM concentration were tested against agitated cultures of *B. subtilis* cells (~10^8 CFU/ml) under robust growth. In the spot assays, the lytic agents are spotted on *B. subtilis* lawns seeded with ~10^6 CFU/ml and growth allowed to occur overnight, under static conditions, until stationary phase. As explained before, these conditions (static slow growth) are expected to increase the bacterial susceptibility to LysSPP1, somewhat resembling the bacteriolytic assays under static conditions. In addition, spotting a small volume of a 1 µM enzybiotic solution, which was the highest concentration tested against *B. subtilis*, will certainly result in a lower local concentration of the lytic agent as result of protein diffusion and dilution through the agar lawn. This might also have contributed to the lower AMPLys activity if we consider the 1 µM threshold concentration for observing a Smap-mediated enhancement of LysSPP1 activity. Actually, the addition of the Smap peptide seems to inhibit LysSPP1 activity when evaluated by the spot-on-lawn assay; this was particularly evident for the SmapLysSPP1 fusion (see Figure **III.11** MICs of LysSPP1 and its AMPLys derivatives on B. subtilis and S. aureus.**Figure III.11A**). The reasons for the Smap inhibitory effect in this particular assay conditions are unknown. One possibility could be a lower stability of the AMPLys during the long incubation period (overnight) of the spot-on-lawn assay.

Besides the work here described, there is only one study that has explored the addition of a specific peptide to an endolysin from a Gram-positive system, as a new approach to improve its lytic and antibacterial properties (Rodríguez-Rubio *et al*., 2016). In the referred study, the authors performed the 'artilysation' of the streptococcal endolysin λSa2lys by fusing to the enzyme's C-terminus a polycationic nonapeptide (PCNP, with aa sequence KRKKRKKRK). The resulting fusion, Art-240, caused a reduction of bacterial viability that was 0.5 to 2.0 log units higher than that of the native endolysin, depending on the target strain and time of cell/Art-240 contact. Art-240 activity displayed also increased tolerance to high salt concentrations and pH variations (up to 3.5 CFU log reduction after 1 hr incubation with 0.2 µM Art-240 under optimal conditions). Of note, with exception of the spot-on-lawn assays, all the experiments used to evaluate the bacteriolytic and bactericidal activity of Art-240 were performed with washed and buffer-resuspended cells. It is therefore unknown if the superior Art-240 antibacterial activity is maintained when tested against bacteria actively growing in complex media.

To explain the improved performance of Art-240 the authors speculated that the positive charges of PCNP, which is made exclusively of arginine and lysine residues, could facilitate the interactions of the enzybiotic with the polyanionic cell surface (negative charges derived from the phosphate groups of teichoic acids associated to the CW) (Rodríguez-Rubio *et al*., 2016). Previous results that showed a positive correlation between the number of positive charges in CDs or CWBDs and lytic activity (Díez-Martínez *et al*., 2013; Low *et al*., 2011) seemed to support this hypothesis. The high PCNP pI and the predicted stronger electrostatic interactions would also fit the increased enzymatic activity over broader pH and salt ranges. At first glance these arguments could also be used to explain the improved performance of our AMPLys fusions, since the 17 amino acid long Smap peptide possesses 10 arginine/lysine residues. However, the ensemble of our results is not consistent with an increase of lytic activity as result of a Smap-mediated improvement of the affinity towards the cell surface. Particularly, in such scenario our most active AMPLys, LysSPP1Smap, would have outperformed the native endolysin at any tested concentration, as described for Art-240 (Rodríguez-Rubio *et al*., 2016). The fact that the much higher bacteriolytic/bactericidal activity of LysSPP1Smap was only revealed at ≥1 μM concentration is suggestive of a mechanism that depends on the amphipathic nature of Smap (and not only on its charged character) and its activity as an AMP. Specifically, we believe that after local accumulation of a certain amount of LysSPP1Smap on the cell surface, the AMP nature of Smap causes a perturbation in the ionic environment of the CW, which might involve its insertion in the cytoplasmic membrane. Such sudden perturbation in the ionic and/or energy state of the cell envelope has been shown to greatly enhance bacterial susceptibility to endolysins (Fernandes & São-José, 2016; Proença *et al*., 2015).

Overall, our results indicate that equipping endolysins with AMP-derived peptides like Smap may contribute to a significant increase of their antimicrobial activity against Gram-positive bacteria growing in complex media. This strategy can therefore be followed to enhance the antibacterial potency of endolysins under clinically relevant contexts, aiming its future development as therapeutic agents.

V. CONCLUDING REMARKS

This project allowed the development of fusion proteins as members of a new class of enzybiotics, the AMPLys, here proposed to have enhanced antibacterial activity against Gram-positive bacteria under growth-promoting conditions. The rationale behind AMPLys development is that the coupling of the PMF-disturbing properties of AMPs to endolysins will boost the lytic activity of the phage enzymes when applied externally to cells growing in complex media. This enhancement of endolysins lytic activity may result in a better therapeutic performance of these agents when applied as enzybiotics *in vivo*. The construction and antibacterial activity of the Smap-LysSPP1 fusions described in this work opens good perspectives for the development of this new class of enzybiotics. As future work, it will be interesting to test AMPLys fusions designed to target well-known pathogens, like for example multidrug-resistant staphylococcal, streptococcal and enterococcal species. Certainly, it will be most important to test these new AMPLys in animal models of infection to validate their superior performance and safety.

VI. MATERIALS AND METHODS

VI. 1. Bacterial Strains, Culture Media and Growth Conditions

Bacterial cells were routinely grown in LB medium (Sambrook & Russell, 2001) under aerated conditions (orbital shaking at 200 rpm). The *E. coli* cloning strain XL1-Blue MRF' (Stratagene) and the expression strains C41(DE3) and C43(DE3) (Miroux & Walker, 1996) were grown at 37ºC. The *E. coli* expression strain BL21/pGP1-2 (also known as CG61, São-Jose *et al.*, 2000) was grown at 30ºC. Cells were pre-cultured overnight (ON) and then diluted (1:50 or 1:100) in fresh LB medium. For plasmid selection LB medium was supplemented with ampicillin (100 μg/ml) and/or kanamycin (40 μg/ml) or with chloramphenicol (20 μg/ml). *B. subtilis* strain YB886 and *S. aureus* strain RN4220 were used to assay the lytic action of the endolysin-derived products. Both *B. subtilis* and *S. aureus* cells were pre-cultured ON in LB medium at 30ºC, with aeration. When necessary, 1.4% or 0.7% agar was added to LB medium to obtain solid or soft-agar plates, respectively. Culture media components were purchased from Biokar Diagnostics (Beauvais, France) or AppliChem (Darmstadt, Germany). Specific culture conditions for protein expression are detailed in section VI.3.

VI. 2. Construction and Cloning of AMP-Endolysin Fusion Genes

The coding sequences of the selected AMPs (Salusin-β and Smap) and flanking flexible linkers (see Supplementary Material) were optimized to the *E. coli* codon usage (web tool OPTIMIZER, Puigbo *et al.*, 2007). These sequences were PCR-amplified using self-annealing oligonucleotides (listed in Supplementary Table S1) and the high-fidelity DNA Polymerase KOD Hot Start Master Mix (Novagen). PCR amplification conditions were determined considering the annealing temperature of primers, size of amplification product and the recommendations of the polymerase manufacturer. The primers used in each AMP amplification carried a 29/30-bp complementary segment in the 3' end, which allowed their annealing and amplification by the Overlap-Extension PCR (OE-PCR) technique. The forward and reverse primers of each pair carried in the 5' end the *Xma*I/*Nco*I and *Nco*I/*Xma*I restriction sites, respectively. This allowed cloning and fusing the AMP coding sequence either upstream (*Nco*I cut) or downstream (*Xma*I cut) the LysSPP1 gene carried in pIV::25His (Fernandes and São-José, 2016). Plasmid pIV::25His is a derivative of the expression vector pIVEX2.3d (Roche Applied Science), which allows expression of cloned genes under the control of phage T7 ϕ10 promoter and the production of the corresponding proteins C-terminally fused to a hexahistidine tail. The AMP PCR products were purified using the *NZYGelpure* (NzyTech Genes & Enzymes) or *GeneJet PCR Purification Kit* (Thermo Scientific), digested either with *Nco*I or *Xma*I and ligated to the equally digested pIV::25His using T4 DNA ligase (Thermo Scientific). *E. coli* strain XL1-Blue MRF' was transformed with the resulting ligations as described by Chung *et al.*, (1989), and transformants selected in the presence of amp (100 μg/ml). The screening for the presence of the desired recombinant plasmids was carried out by PCR, using vector and insert complementary primers (listed in Supplementary Table S1) and DNA Polymerase GoTaq G2 Green Master Mix (Promega) or NZYTaq 2x Green Master Mix (NzyTech Genes and Enzymes). Recombinant plasmids with the desired structure were extracted and purified using *NzyMiniprep* (NzyTech Genes and Enzymes) or *GeneJet*

Plasmid Miniprep Kit (Thermo Scientific) and their sequence confirmed by DNA sequencing (GATC Biotech, Cologne, Germany).

VI. 3. Production and Purification of Protein Fusions

The pIVEX2.3d derivatives pIVEX2.3d::LysSPP1Salβ, pIVEX2.3d::SalβLysSPP1, pIVEX2.3d::LysSPP1Smap and pIVEX2.3d::SmapLysSPP1 were used to transform *E. coli* strain CG61, which produces the phage T7 RNA polymerase upon temperature up-shift. Transformants were selected at 28ºC in the presence of ampicillin (100 μg/ml) and kanamycin (40 μg/ml). The recombinant plasmids pIVEX2.3d::LysSPP1Salβ and pIVEX2.3d::SalβLysSPP1 were also used to transform *E. coli* strains C41(DE3) and C43(DE3), which express the T7 RNA polymerase upon IPTG (0.5 mM) induction. These strains carried also the plasmid pLacI that constitutively produces the LacI repressor for tight regulation of T7 RNA polymerase expression. C41(DE3) and C43(DE3) derivatives were selected at 37ºC in the presence of ampicillin (75 μg/ml) and chloramphenicol (20 μg/ml).

VI. 3.1. Production of Salusin-β-endolysin fusions

Different conditions for Salusin-β-endolysin production were tested to try optimizing protein yield and solubility.

In a first attempt, CG61 derivatives expressing each endolysin fusion (LysSPP1Salβ and SalβLysSPP1) were grown at 28ºC until mid or late exponential growth phase (optical density at 600nm (OD_{600nm}) of 0.5-0.6 and 0.8-1.0, respectively), after which protein production was induced by incubating the cultures in a shaking water bath set to 42ºC. After 30 min induction, cultures were transferred to an orbital incubator at 37ºC and growth maintained during the indicated time. Induced cultures were pelleted by centrifugation (5,400 *g*, 20 min, 4ºC) and resuspended in 1/50 volumes of lysis buffer (50 mM Hepes, 500 mM NaCl, 1% Glycerol, 0.1% triton X-100, pH 7.0) supplemented with 1x Complete EDTA-free Protease Inhibitor Cocktail (Roche Applied Science). Cells were kept on ice and disrupted by sonication (Vibra Cell, Sonics Materials) with 5 to 7 bursts of 15 sec (amplitude 50%, pulse 5, 20-30 W) and with 45 sec pauses between bursts. Crude protein extracts were cleared by centrifugation (12,500 *g*, 30 min, 4ºC). The pelleted insoluble material was solubilized in a thiourea-urea buffer (7 M urea, 2 M thiourea, 50 mM Tris, pH 8.0). For producing small scale protein extracts, cells from 1 ml culture samples were 20-fold concentrated in the commercially available lysis buffer *Complete lysis-B (2x) EDTA-free* (Roche Applied Science), supplemented with 1x Complete EDTA-free Protease Inhibitor Cocktail (Roche Applied Science), and soluble and insoluble material prepared as described above.

In a second approach, C41(DE3) and C43(DE3) derivatives expressing the same protein fusions were grown at 37ºC, until an OD_{600nm} of 0.5-0.6. Protein production was induced with 0.5 mM of IPTG and cells further incubated for 2 hr. Preparation of protein extracts was as described above.

In a final attempt, we used as culture medium LB buffered with 0.1 M sodium phosphate (pho-Na buffer, pH 7.2) and supplemented either with 0.5 M D-sorbitol or 0.2 M L-Arginine monohydrochloride (L-Arg•HCl). For preparation of these media, 2-fold concentrated LB was supplemented with 1 M D-sorbitol or with 0.4 M L-Arg•HCl and autoclaved. These were then mixed 1:1 with 0.2 M pho-Na buffer that had been separately prepared and autoclaved. Protein production in this altered culture medium was

attempted with the CG61 and C41(DE3) derivatives. Cells were grown until OD_{600nm} of ~0.5 at 28ºC (CG61 derivatives) or 37ºC (C41(DE3) derivatives)) and protein production induced by temperature up-shift or 0.5 mM IPTG addition, respectively. Induced cultures in presence of 0.5 M D-sorbitol were incubated for 16 hr at 16ºC, in a shaking water bath, while those supplemented with 0.2 M L-Arg•HCl were incubated at 37ºC for 30 min. Protein extraction was as described above.

VI. 3.2. Production and purification of Smap-endolysin fusions

CG61 derivatives carrying pIVEX2.3d::LysSPP1Smap or pIVEX2.3d::SmapLysSPP1 were used to produce the fusions SmapLysSPP1 and LysSPP1Smap. Culture conditions were as described for the first attempt of Salusin-β-endolysin production (see VI.3.1), except that induction of protein production was carried out when cultures reached an OD_{600nm} of 0.6, and protein synthesis allowed to occur for 2 hr after changing cultures from 42 to 37ºC. Cells were pelleted by centrifugation (5,400 *g*, 15 min, 4ºC) and resuspended in 1/50 volumes of lysis buffer A (50 mM Hepes, 500 mM NaCl, 0.1% triton X-100, 1% glycerol, 50 mM Imidazole, pH 7.0) supplemented with 1x Complete EDTA-free Protease Inhibitor Cocktail (Roche Applied Science) and 10 µg/ml DNAse A. Cells lysis through sonication and elimination of insoluble material was as described above. The total soluble protein extracts were filtered through a 0.22 µm membrane and the fusion proteins purified by affinity chromatography (AF) using *HisTrap™ HP* columns (GE Healthcare Life Scinces), coupled to an ÄKTA-Prime system (*ÄKTAprime™ plus*, GE Healthcare Life Sciences). The column and elution buffer had the same composition of lysis buffer A (without supplements), except that the elution buffer had a concentration of imidazole of 500 mM. After the AF step, partially purified proteins were subjected to size-exclusion chromatography (SEC) using a HiLoad 16/60 superdex 75 prep grade column (GE Healthcare Life Sciences), also coupled to ÄKTA-Prime system, which was equilibrated and run with imidazole-free protein buffer P (50 mM Hepes, 500 mM NaCl, 1% Glycerol, 0.1% triton X-100, pH 7.0). Fractions of purified proteins were pooled and concentrated using Vivaspin concentrators with a cut-off of 10,000 MW (Sartorius Stedim Biotech), according to the manufacturer recommendations. Protein concentrations were determined by the Bradford method (Bio-Rad Laboratories), using bovine serum albumin (BSA) as standard. The enzymes were divided in small aliquots and kept at -80ºC.

VI. 3.3. SDS-PAGE and western blot analysis

Protein production and purification was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis as described elsewhere (Sambrook & Russell, 2001). His_6-tagged proteins were immunodetected either with an Anti-His_6-Peroxidase mouse monoclonal antibody (Roche Applied Science) or with an Anti-His_6 mouse monoclonal primary antibody (Roche Applied Science) and a secondary anti-mouse IgG-POD antibody (Roche Applied Science). Antigen/antibody complexes were revealed with *BM Chemiluminescence Western Blotting Kit (Mouse/rabbit)* (Roche Applied Science).

VI. 4. Evaluation of the Antibacterial Activity of Smap-Endolysin Fusions

VI. 4.1. Bacteriolytic activity of SmapLysSPP1 and LysSPP1Smap

The ability of SmapLysSPP1 and LysSPP1Smap to induce lysis of bacterial cells in liquid medium was evaluated under static or aerated conditions (culture flaks with orbital shaking, 180 rpm), in both cases with cultures maintained at 37ºC. The native LysSPP1 (available lab stock, Fernandes & São-José, 2016) was used as comparative in these assays and in all other experiments described below. To study lysis under static conditions, bacterial strains were grown by the standard procedure (see above) until OD_{600nm} of 0.8 (*B. subtilis* and *S. aureus*) or 0.5 (*E. coli* strains TG1 and BL21 (Stratagene)), they were distributed in 1-ml spectrophotometer cuvettes, and then challenged with the indicated concentrations of the lytic agents (equivalent volumes of protein buffer used as negative controls). Cell lysis was monitored by taking regular OD_{600nm} measurements over a period of 60 min. For the lysis assays under aerated conditions (only studied with *B. subtilis* YB886), cell cultures were maintained under agitation after addition of the lytic agents. Lysis was monitored by taking 1-ml culture samples at regular intervals for OD_{600nm} determination.

VI. 4.2. Bactericidal activity of SmapLysSPP1 and LysSPP1Smap

The bactericidal activity of the fusion proteins was characterized in terms of their impact on cell viability expressed as colony forming units per milliliter (CFU/ml). *B. subtilis* YB886 cells were grown under standard conditions until OD_{600nm} of 0.8 and then challenged with the indicated concentrations of the agents for 30 min, under aerated conditions, and at 37ºC. Cell death was expressed in terms of log reduction of the CFU/ml values after the 30 min contact with the bactericidal agent. Equivalent volumes of protein buffer were used as negative controls. Analogous cell viability assays were also performed with *S. aureus* RN4220 strain.

VI. 4.3. Minimum inhibitory concentrations of SmapLysSPP1 and LysSPP1Smap in solid medium

The capacity of the lytic agents to inhibit growth of *B. subtilis* YB886 and *S. aureus* RN4220 strains was evaluated in soft-agar LB plates. Cells were grown under standard conditions until OD_{600nm} of 0.8 for YB886, and 0.6 for RN4220. A sample of the target bacteria (~10^7 CFU) was incorporated in 10 ml LB soft-agar and poured on a Petri dish (~10^6 CFU/ml final concentration). After medium solidification, plates were inverted and dried at 37ºC for 30 min with the lids partially opened. Then, different protein concentrations (ranging from 7.81 nM to 4 µM in a final volume of 5 µl) were spotted on the bacterial lawns. The lytic agent concentration series were prepared by sequential 2-fold dilutions, with the highest concentration prepared in LB medium. Dilutions were performed in LB medium supplemented with the equivalent amount of protein buffer of the highest concentration. This ensured even composition of the samples, expect for the protein amount. The spots were allowed to dry and plates incubated ON at 37ºC in inverted position. Growth inhibition was evaluated according to relative size and transparency of the growth inhibition halos. Negative controls were prepared by spotting endolysin dilution buffer instead of endolysin.

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VIII. SUPPLEMENTARY MATERIAL

LysSPP1SalB

MGKLVWLDAGHGGKDSGAAANGIKEKDIVLKIVKKVKSILTSRYEVAVKLTRDSDVFYELIDRA
RKANAAKADLFVSVHINATPGGKGFETYRYVKTSASSSTGQQQKVLHDAIYKRIKKYGIKDRGE
KAADLSVLRNTSMPAVLTENLFIDNKDEAALLKKDSFLNDVAEGHAEGIAEILNLKKKSGGSAP
KKEDKPSSGKTKMVIVKDNPDGFLWVYNKADWNARYKKVKPDEAFTIDKTVTVNGSKMYKLKSG
LYITAASKYVTVKEKPGGGGSMAIFIFIRWLLKLGHHGRAPPGGGSPGGGSHHHHHH

SalBLysSPP1

MAIFIFIRWLLKLGHHGRAPPGGGSPGSMGKLVWLDAGHGGKDSGAAANGIKEKDIVLKIVKKV
KSILTSRYEVAVKLTRDSDVFYELIDRARKANAAKADLFVSVHINATPGGKGFETYRYVKTSAS
SSTGQQQKVLHDAIYKRIKKYGIKDRGEKAADLSVLRNTSMPAVLTENLFIDNKDEAALLKKDS
FLNDVAEGHAEGIAEILNLKKKSGGSAPKKEDKPSSGKTKMVIVKDNPDGFLWVYNKADWNARY
KKVKPDEAFTIDKTVTVNGSKMYKLKSGLYITAASKYVTVKEKPGGGSHHHHHH

LysSPP1Smap

MGKLVWLDAGHGGKDSGAAANGIKEKDIVLKIVKKVKSILTSRYEVAVKLTRDSDVFYELID
RARKANAAKADLFVSVHINATPGGKGFETYRYVKTSASSSTGQQQKVLHDAIYKRIKKYGIK
DRGEKAADLSVLRNTSMPAVLTENLFIDNKDEAALLKKDSFLNDVAEGHAEGIAEILNLKKK
SGGSAPKKEDKPSSGKTKMVIVKDNPDGFLWVYNKADWNARYKKVKPDEAFTIDKTVTVNGS
KMYKLKSGLYITAASKYVTVKEKPGGSMARKLRRLKRKIAHKVKKYGPGGGSHHHHHH

SmapLysSPP1

MARKLRRLKRKIAHKVKKYGPGSMGKLVWLDAGHGGKDSGAAANGIKEKDIVLKIVKKVKS
ILTSRYEVAVKLTRDSDVFYELIDRARKANAAKADLFVSVHINATPGGKGFETYRYVKTSA
SSSTGQQQKVLHDAIYKRIKKYGIKDRGEKAADLSVLRNTSMPAVLTENLFIDNKDEAALL
KKDSFLNDVAEGHAEGIAEILNLKKKSGGSAPKKEDKPSSGKTKMVIVKDNPDGFLWVYNK
ADWNARYKKVKPDEAFTIDKTVTVNGSKMYKLKSGLYITAASKYVTVKEKPGGGSHHHHHH

Figure S1. – Schematic representation of the domains and flanking segments composing the AMPLys fusions. The schemes are not at scale. Details of the amino acidic sequences are provided below with the corresponding colour code of the scheme.

Table S1. Primers sequences used in this study.

Name	5'→3' sequence
SalBeta-Fw	CGA**CCCGGG**GGCGGTGGCT**CCATGG**CGATTTTTATCTTCATT**CGT TGGCTGCTGAAACTGGGTCATCACGGT**
SalBeta-Rv	CGA**CCATGG**A**CCCGGG**GCTGCCACCGCCCGGCGGCGCACG**ACCG TGATGACCCAGTTTCAGCAGCCAACG**
SMAP-Fw	TATCGA**CCCGGG**GGTT**CCATGG**CGCGTAAGCT**GCGTCGTCTGAA ACGTAAGATCGCGCACA**
SMAP-Rv	TATCGA**CCATGG**A**CCCGGG**GCCGTATTTCTTCACTT**TGTGCGCGA TCTTACGTTTCAGACGACGC**
pIVEX-Rev	ATTGCTCAGCGGTGGCAG
T7p	TAATACGACTCACTATAGGG
LysSPP1trc	GTCGAATTCAGCTTGAAGCACTCGTTTTCACA

Bold case sequences correspond to restriction sites used for cloning: CCCGGG: *Xma*I; CCATGG: *Nco*I. Bold and underlined sequences referrer to the complementary sequences of the primers used to the OE-PCR technique.